Universidad Pública de Navarra

Novel application of bioassays and functional importance of minor crystal components of *Bacillus thuringiensis* serovar *israelensis* for dipteran pests

DANIEL VALTIERRA DE LUIS

Pamplona, 2019

Universidad Pública de Navarra

Departamento de Agronomía, Biotecnología y Alimentación

Novel application of bioassays and functional importance of minor crystal components of *Bacillus thuringiensis* serovar *israelensis* for dipteran pests

Tesis Doctoral para optar al grado de Doctor, presentada por:

Daniel Valtierra de Luis

Directores:

Dr. Primitivo Caballero Murillo

Dra. Maite Villanueva San Martín

Pamplona, 2019

Miembros del tribunal

Presidente

Dr. Colin Berry
School of Bioesciences
Cardiff University

Suplente de presidente

Dr. Jesus Murillo Martínez
Departamento de Agronomía,
Biotecnología y Alimentación
Universidad Pública de Navarra

Secretaria

Dra. Mª Pilar Medina Vélez
Departamento de Producción Agraria
Universidad Politécnica de Madrid

Suplente de secretaria

Dra. Flor Budia Marigil
Departamento de Producción Agraria
Universidad Politécnica de Madrid

Vocal

Dra. Susana Vílchez Tornero
Departamento de Bioquímica
Universidad de Granada

Suplente de Vocal

Dr. Javier Lucientes Curdi
Departamento de Patología Animal
Universidad de Zaragoza

Revisores externos

Titulares

Dr. Colin Berry
School of Bioesciences
Cardiff University

Dra. Flor Budia Marigil
Departamento de Producción Agraria
Universidad Politécnica de Madrid

Suplentes

Dra. Susana Vílchez Tornero
Departamento de Bioquímica
Universidad de Granada

Dra. Mª Pilar Medina Vélez
Departamento de Producción Agraria
Universidad Politécnica de Madrid

Dr. PRIMITIVO CABALLERO MURILLO
Catedrático de Producción Vegetal del Departamento de Agronomía, Biotecnología y Alimentación de la Universidad Pública de Navarra.

Dra. MAITE VILLANUEVA SAN MARTÍN
Investigadora Doctora, Área de Producción Vegetal del Departamento de Agronomía, Biotecnología y Alimentación de la Universidad Pública de Navarra.

INFORMAN,

Que la presente memoria de Tesis Doctoral "Novel application of bioassays and functional importance of minor crystal components of *Bacillus thuringiensis* serovar *israelensis* for dipteran pests" elaborada por **Daniel Valtierra de Luis** bajo su dirección, cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctor.

Y para que así conste, firma la presente en Pamplona/Iruña, a 16 de septiembre de 2019.

Fdo. Primitivo Caballero Murillo

Fdo. Maite Villanueva San Martín

"Toma riesgos y prepárate para fallar"

Ed Catmull

"Que algo no haya salido como hayas querido no significa que sea inútil"

Thomas Edison

Agradecimientos

En primer lugar, me gustaría agradecer a mi director de Tesis, **Primitivo Caballero**, por darme la oportunidad de realizar la tesis Doctoral en su grupo de investigación. También agradecer el que haya posibilitado exponer los resultados obtenidos en congresos tanto nacionales como internacionales.

A **Maite Villanueva**, mi co-directora. Desde que llegó al laboratorio nos entendimos perfectamente, empezamos a trabajar mano a mano, y realmente me considero afortunado de haberla tenido a mi lado durante estos años.

A **Trevor Williams**, con el que he tenido la oportunidad de poder trabajar y aprender al publicar muchos de los resultados que se han obtenido durante la realización de este trabajo.

A todas las personas que han contribuido a la realización de este trabajo. En especial, a **Javier**, por las interminables conversaciones/terapias, y por las magníficas fotos que aparecen en esta Tesis. A **Adriana**, por su apoyo moral y por su gran ayuda a la hora de maquetar el documento final. A **López**, que me ayudó en la corrección idiomática de la introducción. A todos los miembros del laboratorio con los que he convivido durante estos años: **Edu, Mikel, Belda, Argine, Mireya, Inés, Ana y Quique**. A aquellas personas que forman parte del centro de investigación y con los que he compartido buenos momentos: Víctor, Rosa, Iñigo, Oihane, Ale, Arancha, Naiara, Leti, Pilar, Damián, Ramsés, Lorena, Ana Z…

A todas las personas que desarrollan y promueven el software libre, en especial a:

> **Alexandra Asanovna Elbakyan**, neurocientífica y desarrolladora de software, que en el año 2011 fundó **Sci-Hub**, repositorio y página web que brinda una gran cantidad de artículos científicos de manera gratuita, sin suscripción ni pagos, posibilitando realmente la difusión del conocimiento científico, y a mí en particular a tener acceso a gran cantidad de artículos que de otro modo no sería posible.
>
> **Robert Gentleman**, **Ross Ihaka** y posteriores desarrolladores (***R Development Core Team***) del proyecto **R software**, entorno de software libre para computación estadística y gráficos que me ha permitido realizar el análisis de datos durante la realización de esta tesis doctoral sin la dependencia de otros softwares de pago.

L@s desarroladores del gestor bibliográfico gratuito **Mendeley** que, gracias a este, he podido realizar todo el trabajo bibliográfico que conlleva una tesis doctoral.

A mi **Madre** y a mi **Hermana**, que siempre están ahí en los buenos y malos momentos y a las que debo todo.

A **Adriana**, mi compañera de vida, la que me da fuerza y alegría día a día.

A mis amigos: **Mayans, Jose, Javi, López, Manuel, Jaouad** por su apoyo incondicional en todas las facetas de la vida.

Al **JiuJitsu**, mi pasión, que me ayuda diariamente a dar lo mejor de mí.

Gracias a Tod@s!!

TABLE OF CONTENTS

RESUMEN

RESUMEN

Bacillus thuringiensis (Berliner, 1915) (Bt) es una bacteria Gram-positiva de forma redondeada que tiene la capacidad de formar esporas de resistencia. Bajo condiciones desfavorables, la bacteria esporula produciendo la espora y el cristal paraesporal. Este cristal está compuesto principalmente por una o más proteínas insecticidas, también llamadas δ-endotoxinas, las cuales son capaces de formar inclusiones cristalinas. Los insecticidas basados en Bt representan alrededor del 80% de todos los bio-plaguicidas existentes en el mercado. La toxicidad específica de las proteínas del cristal contra insectos diana es la base para el empleo de Bt como bioplaguicida en agricultura, selvicultura y control de mosquitos desde 1961. Actualmente la alternativa más importante para el control de larvas de mosquito y mosca se basa en toxinas producidas por *B. thuringiensis* ser. *israelensis* (Bti).

Son necesarios métodos de bioensayo simples y reproducibles para evaluar la toxicidad de nuevos compuestos insecticidas para el manejo de plagas y para determinar la presencia de resistencias en poblaciones plaga. Hemos utilizado un isótopo radioactivo basado en ^{32}P-ATP para estimar el volumen ingerido por dos plagas de dípteros: *Ceratitis capitata* (Tephritidae) y *Drosophila suzukii* (Drosophilidae). Utilizando un colorante azul en el tratamiento, fue posible distinguir individuos que habían ingerido la solución de aquellos que no lo había hecho. El volumen medio ingerido por adultos de *C. capitata* fue 1.968 µl. Las hembras ingirieron aproximadamente un ~20% más de volumen que los machos. Los adultos de *D. suzukii* ingirieron una media de 0.879 µl, ingiriendo las hembras aproximadamente un ~30% más que los machos. El método de la gota fue validado utilizando como ingrediente activo (i.a.) el plaguicida de origen natural spinosad. En *C. capitata*, la respuesta concentración-mortalidad no varió entre sexos, ni entre tres lotes distintos de insectos. Los valores de dosis letal fueron calculados basándonos en el volumen medio ingerido. En *C. capitata* los valores de LD_{50} fueron 1.462 and 1.502 ng i.a./insecto para machos y hembras respectivamente. Cuando se consideró la variación específica del peso corporal dependiente de sexo, obtuvimos valores de 0.274 and 0.271 ng i.a./mg para machos y hembras respectivamente. Empleamos el mismo método para determinar los valores de *D. suzukii*. Esta técnica puede ser directamente empleada para determinar

los estados de resistencia y la respuesta dosis-mortalidad de un compuesto insecticida en muchas especies de dípteros plaga.

Bacillus thuringiensis ser. *israelensis* (Bti) forma cuerpos de inclusión paraesporales con forma semiesférica compuestos de proteínas insecticidas, representadas principalmente por cinco familias (Cry4, Cry10, Cry11, Cyt1 and Cyt2), los cuales son ampliamente utilizados como larvicidas microbianos frente a plagas de dípteros. La actividad insecticida del cristal completo es alta si se compara con las actividades de las proteínas individuales, probablemente debido a las interacciones sinérgicas que existen entre las distintas proteínas del cristal, especialmente aquellas que involucran a Cyt1Aa. En este estudio se clonaron Cry10Aa y Cyt2Ba a partir del producto comercial con actividad larvicida VectoBac® 12AS y se expresaron en la cepa de Bt acristalófora BMB171. Estas proteínas mostraron valores de LC_{50} de 299.62 y 279.37 ng/ml, respectivamente, frente a larvas de segundo estadio de *A. aegypti*, pero no mostraron actividad tóxica frente a adultos de *C. capitata*. Cuando las larvas de *A. aegypti* ingirieron una mezcla equitativa (ratio 1:1) de esporas y cristales de las proteínas recombinantes Cry10Aa y Cyt2Ba, se observó una fuerte actividad sinérgica. Se estimó que la magnitud de este efecto sinérgico fue de 34.3, encontrándose entre los valores más altos descritos hasta el momento para los componentes del cristal de Bti, y comparable a los publicados previamente para Cyt1A con Cry4A y Cry11A. Por lo tanto, los componentes minoritarios de Bti tienen una relevancia en la toxicidad global de la cepa y ambas proteínas pueden desempeñar un papel importante en la sinergia y en la prevención de la aparición de resistencias.

SUMMARY

SUMMARY

Bacillus thuringiensis (Berliner, 1915) (Bt) is a Gram-positive rod-shaped bacterium with the capacity to form resistance spores. Under unfavourable conditions, the bacterium sporulates producing the spore and the parasporal body. The latter is primarily composed by one or more insecticidal proteins, also called δ-endotoxins, which are able to form crystalline inclusions. Bt-based insecticides represent around 80% of all biopesticides in the market. The specific toxicity of crystal proteins against target insects is the basis for the use of Bt as a biopesticide in agriculture, forestry and mosquito control since 1961. Currently, the major alternative for mosquito and blackfly larval control is based on bacterial toxins produced by *B. thuringiensis* ser. *israelensis* (Bti).

Simple and repeatable bioassay methods are required to evaluate the toxicity of novel insecticidal compounds for pest management and to determine the presence of resistance traits in pest populations. We used a radioactive tracer based on ^{32}P-ATP to estimate the volume ingested by two dipteran pests: *Ceratitis capitata* (Tephritidae) and *Drosophila suzukii* (Drosophilidae). Using blue food dye it was possible to distinguish between individuals that ingested the solution from those that did not. The average volume ingested by *C. capitata* adults was 1.968 µl. Females ingested a ~20% greater volume of solution than males. Adults of *D. suzukii* ingested an average of 0.879 and females ingested ~30% greater than males. The droplet feeding method was validated using the naturally-derived insecticide spinosad as the active ingredient (a.i.). For *C. capitata*, the concentration-mortality response did not differ between the sexes or among three different batches of insects. Lethal dose values were calculated based on mean ingested volumes. For *C. capitata* LD_{50} values were 1.462 and 1.502 ng a.i./insect for males and females, respectively, equivalent to 0.274 and 0.271 ng a.i./mg for males and females respectively, when sex-specific variation in body weight was considered. We use the same process for determining the *D. suzukii* values. This technique could be readily employed for determination of the resistance status and dose-mortality responses of insecticidal compounds in many species of dipteran pests.

B. thuringiensis ser. *israelensis* forms semi-spherical parasporal inclusion bodies composed of insecticidal proteins, mainly represented by five families (Cry4, Cry10, Cry11, Cyt1 and Cyt2), which

are widely used as the basis for microbial larvicides against several dipteran pests. The insecticidal activity of the crystal is high in comparison to the activities of the individual toxins, which is likely due to synergistic interactions among the crystal proteins, particularly those involving Cyt1Aa. In the present study Cry10Aa and Cyt2Ba were cloned from the commercial larvicide VectoBac® 12AS and expressed in the acrystalliferous Bt strain BMB171. These proteins had LC_{50} values of 299.62 and 279.37 ng/ml, respectively, against *A. aegypti* second instars but did not show toxic activity against *C .capitata* adults. When the *A. aegypti* larvae ingested an equitable mixture (ratio 1:1) of spores and crystals of the Cry10A and Cyt2B recombinant proteins, a strong synergistic activity was observed. It was estimated that the magnitude of this synergistic effect was 34.3, which is among the highest values described so far for the components of the Bti crystal and comparable to those previously reported for Cyt1A with Cry4 and Cry11A. Therefore, Bti minor components have relevance in the overall toxicity of the strain and both proteins can play an important role in synergy and resistance avoidance

CHAPTER I

Introduction

TABLE OF CONTENTS

1. THE ENTOMOPHATOGENIC BACTERIA *Bacillus thuringiensis*

Since its discovery, in 1901, and correct scientific description, in 1915, *Bacillus thuringiensis* (Bt) has been isolated from the most diverse habitats of our planet (Iriarte et al., 1998; Martin and Travers, 1989). This has led to the characterization of a large number of Bt strains that, as a whole, have revealed an enormous genetic diversity of this bacterium. This genetic diversity corresponds in good measure to the multiple functions that this bacterium plays in natural and transformed ecosystems (agricultural and forestry). Some of the most relevant functions attributed to Bt from the applied point of view are: plant growth-promoting activities (PGPR), bioremediation of different heavy metals and other pollutants, biosynthesis of metal nanoparticles, production of polyhydroxy alkanoate biopolymer, and anticancer activities (Jouzani et al., 2017). In agriculture it is, without a doubt, the most popular bacterium because of its usefulness as a biological pest control agent and to be the most important source of insecticidal genes for the construction of resistant transgenic plants (also known as resistant Bt plants) to some of the most important agricultural and forestry pests (Caballero and Ferré, 2001). Bt is also an efficient biological control agent for insect vectors (mainly mosquitoes) of diseases of importance both in the field of human and veterinary health (Federici, 1999).

1.1 Discovery of Bt

Bacillus thuringiensis (Bt) was first discovered in Japan by Shigetane Ishiwata (1901), as the causal agent of sotto disease in *Bombyx mori* larvae (Ishiwata, 1901). He named it Sottokin, which means "sudden death bacillus", and described the pathology caused by the bacterium in silkworm larvae (Ishiwata, 1905). From the beginning it was accepted that a toxin was involved in the pathogenicity of Bt; however, this cause and effect relationship would not be established with due scientific rigor until a few years later. The first taxonomic valid description was made by the German bacteriologist Ernst Berliner (1915), who isolated this bacterium from the Mediterranean flour moth (*Anasagasta küehniella*) (Berliner, 1915). He named it *Bacillus thuringiensis* (Bt), which is derived from Thuringia, the German region where this bacterium was isolated (Berliner, 1915).

1.2 General characteristics of Bt

1.2.1 Interspecific classification of Bt

Bt is a Gram positive, rod-shaped bacterium with the capacity to form resistance spores, classified in the family Bacillaceae (Schnepf et al., 1998). Vegetative Bt cells, while sporulating, characteristically form a parasporal crystal composed of proteins that show different biocidal activities (Palma et al., 2014). This crystal or occlusion body, which is formed by a combination of delta-endotoxins (Cry and Cyt), can acquire different forms (bipyramidal, spherical, etc.) and sizes (smaller, equal or greater than the spore) which are usually typical for each wild Bt strain. (Schnepf et al., 1998). All Bt strains are characterized by the ability to form a parasporal crystal and it is the main morphological characteristic that makes it possible to differentiate Bt strains from those of other species that are very close phylogenetically, such as *Bacillus cereus* and *Bacillus anthracis* (Ibrahim et al., 2010; Raymond et al., 2010; Roh et al., 2007). These three species share a series of genetic and ecological characteristics (Daffonchio et al., 2006; Han et al., 2006; Helgason et al., 2000; Schmidt et al., 2011), but they clearly differ in their pathogenicity. Whereas Bt is mainly toxic for insects and completely innocuous for people, higher animals and plants, *B. cereus* is a foodborne opportunistic pathogen and *B. anthracis* causes anthrax disease in humans (Allende, 2016).

1.2.2 Intraspecific classification of Bt

Several methods have been used for the classification of wildtype Bt isolates. However, the useful and well-known method is based on the immunological reaction (agglutination) to the bacterial flagellar antigen (H serotyping) that occurs when specific antisera that are produced in rabbits is used against the flagellar H antigen (de barjac and Bonnefoi, 1962). This method was stablished as a typing method of choice for the characterization within Bt isolates and has been used until date (de Barjac and Frachon, 1990). Though generally serotypes do not contain H antigen variation, some of them have shown to contain few antigen distinct subfactors (Xu and Côté, 2008), which has led to the subdivision of the serotypes into different serological varieties (serovars). To date, there are at least 72 serotypes (antigenic groups) and 82 serovars based on the flagellar antigen (Table 1) (Blackburn et al., 2013; Lecadet et al., 1999). Though generally serotypes do not contain H antigen variation, some of them have displayed to contain few antigen distinct subfactors (Xu and Côté, 2008). Nonetheless, the

differentiation of strains inside the same H-serotype cannot be accomplished by this method (Ibrahim et al., 2010).

Earlier studies found that there was a kind of correlation between the classification of a Bt strain, based on biochemical characteristics or H serotyping, and its toxicity (Dulmage, 1981). The identification of specific toxins produced by a Bt strain can be used to predict the host range, since Bt toxins are specific. A single toxin or strain is mainly active against a limited number of insect species (Jurat-Fuentes and Jackson, 2012). However, this relationship has been proven to be more complex, due to the appearance of many Bt isolates with novel activities or broader host range (de Barjac and Frachon, 1990) and the presence of toxins from Bt strains without an identified target host (Jurat-Fuentes and Jackson, 2012). Hence, different tools including phylogenetic analysis of the bacterium by using the gyrB gene to complement the 16s rRNA gene, determination of number and size of plasmids, characterization of toxic genes, analysis of protein fragments by polyacrylamide gel electrophoresis (SDS-PAGE) and toxicity bioassays may be applied in order to characterize a Bt isolate.

Table 1. Current list of *B. thuringiensis* Serovars (Jurat-Fuentes and Jackson, 2012).

H Antigen	Serovar	Abbreviation	H Antigen	Serovar	Abbreviation
1	*thuringiensis*	THU	30	*medellin*	MED
2	*finitimus*	FIN	31	*toguchini*	TOG
3a, 3c	*alesti*	ALE	32	*cameroun*	CAM
3a, 3b, 3c	*kurstaki*	KUR	33	*leesis*	LEE
3a, 3d	*sumiyoshiensis*	SUM	34	*konkukian*	KON
3a, 3d, 3e	*fukuokaensis*	FUK	35	*seoulensis*	SEO
4a, 4b	*sotto*	SOT	36	*malaysiensis*	MAL
4a, 4c	*kenyae*	KEN	37	*andaluciensis*	AND
5a, 5b	*galleriae*	GAL	38	*oswaldocruzi*	OSW
5a, 5c	*canadensis*	CAN	39	*brasiliensis*	BRA
6	*entomocidus*	ENT	40	*huazhongensis*	HUA
7	*aizawai*	AIZ	41	*sooncheon*	SOO
8a, 8b	*morrisoni*	MOR	42	*jinghongiensis*	JIN
8a, 8c	*ostriniae*	OST	43	*guiyangiensis*	GUI
8b, 8d	*nigeriensis*	NIG	44	*higo*	HIG
9	*tolworthi*	TOL	45	*roskildiensis*	ROS
10a, 10b	*darmstadiensis*	DAR	46	*chanpaisis*	CHA
10a, 10c	*londrina*	LON	47	*wratislaviensis*	WRA
11a, 11b	*toumanoffi*	TOU	48	*balearica*	BAL
11a, 11c	*kyushuensis*	KYU	49	*muju*	MUJ
12	*thompsoni*	THO	50	*navarrensis*	NAV
13	*pakistani*	PAK	51	*xiaguangiensis*	XIA
14	*israelensis*	ISR	52	*kim*	KIM
15	*dakota*	DAK	53	*asturiensis*	AST
16	*indiana*	IND	54	*poloniensis*	POL
17	*tohokuensis*	TOH	55	*palmanyolensis*	PAL
18a, 18b	*kumamotoensis*	KUM	56	*rongseni*	RON
18a, 18c	*yosoo*	YOS	57	*pirenaica*	PIR
19	*tochigiensis*	TOC	58	*argentinensis*	ARG
20a, 20b	*yunnanensis*	YUN	59	*iberica*	IBE
20a, 20c	*pondicheriensis*	PON	60	*pingluonsis*	PIN
21	*colmeri*	COL	61	*sylvestriensis*	SYL
22	*shandongiensis*	SHA	62	*zhaodongensis*	ZHA
23	*japonensis*	JAP	63	*bolivia*	BOL
24a, 24b	*neoleonensis*	NEO	64	*azorensis*	AZO
24a, 24c	*novosibirsk*	NOV	65	*pulsiensis*	PUL
25	*coreanensis*	COR	66	*graciosensis*	GRA
26	*silo*	SIL	67	*vazensis*	VAZ
27	*mexicanensis*	MEX	68	*thailandensis*	THA
28a, 28b	*monterrey*	MON	69	*pahangi*	PAH
28a, 28c	*jegathesan*	JEG	70	*sinensis*	SIN
29	*amagiensis*	AMA	71	*jordanica*	JOR

1.3 Ecology of *B. thuringiensis*

Bt has been isolated from a large number of environments and ecosystems including soil and water samples, storage dust samples, dead insects, deciduous and coniferous leaves, insectivorous mammals and many other sources (Höfte and Whiteley, 1989; Iriarte et al., 1998; Knowles and Dow, 1993; Raymond et al., 2010; Roh et al., 2007). Several extensive Bt screenings projects have reported that the most prolific environment for isolation of Bt strains is dust and materials associated with grain stores and silos (Bernhard et al., 1997; Chaufaux et al., 1997; Iriarte et al., 1998). In these screenings, more than half of the Bt isolates were pathogenic to a number of species of Lepidoptera, Coleoptera and Diptera. The most abundant Bt serovar in any given area varies by geographic region and environment (Martin and Travers, 1989).

1.4 Biological cycle of *B. thuringiensis*

The Bt life cycle is divided into two phases of growing: a vegetative phase and stationary phase (Ibrahim et al., 2010; Marco and Porcar, 2012). When the environment is suitable for growth, the spore germinates producing a vegetative cell that grows and reproduces by binary fission. Cells continue to multiply exponentially until one or more nutrients, such as sugars, amino acids, or oxygen, become insufficient for life. Under these unfavourable conditions, the bacterium sporulates producing the spore and one or more parasporal bodies. The parasporal body is a crystal primarily composed by one or more insecticidal proteins, which aggregate to form crystalline inclusions (Figure 1).

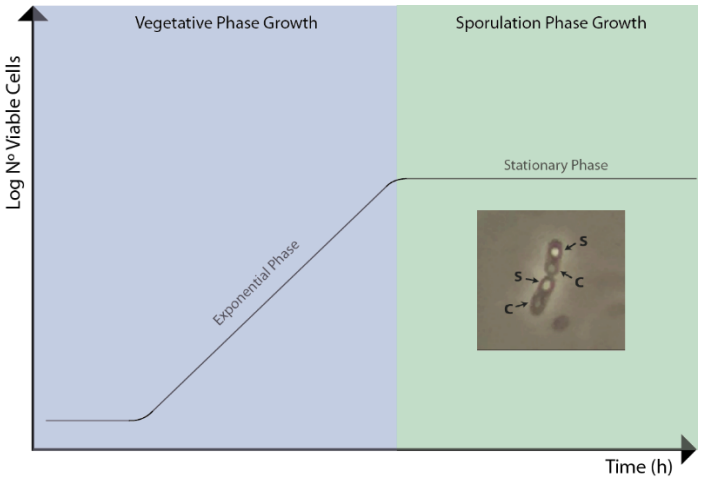


Figure 1. Schematic representation of the life cycle of *Bacillus thuringiensis*. During the vegetative phase of the growth, the bacterium experiences an exponential growth forming collar chains. The lack of nutrients forces the bacterium to enter a stationary phase in which it sporulates and the proteins aggregate to form the parasporal crystal. Finally, the cell lysis releases the crystal and the spore, which will germinate again in favourable conditions, closing the cycle of this bacterium.

1.4.1 Virulence factors produced during the stationary phase

Bt strains characteristically produce a large amount of insecticidal proteins which are specific against a number of insect species from different orders such as Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, Orthoptera, as well as other organisms as mites (Schnepf et al., 1998) and nematodes (Wei et al., 1993). Those proteins aggregate, during the stationary phase, to form one or more parasporal crystals and they are classified into crystal protoxins (Cry) and cytolytic protoxins (Cyt). The genes that code for these proteins (*cry* and *cyt*, respectively) are usually located in native mega plasmids (> 40 mega daltons) (Gonzalez et al., 1981). The size and number of plasmids harbouring these genes are highly variable for each strain and some of them (the conjugative plasmids) can be transferred from a Bt strain to another. The conjugation is a natural way for many bacteria for exchanging genetic information including *cry* or *cyt* genes (Jurat-Fuentes and Jackson, 2012). The shape of the Bt crystals may be variable, depending on the proteins that compose it and the growing conditions of the culture. The most common morphologies are bipyramidal and spherical (Bernhard et al., 1997; Martin and Travers, 1989). The synthesis of the crystal entails a huge metabolic investment from the cell. Moreover, the high protein expression levels that occur in the stationary

growth phase are controlled at the transcriptional, post-transcriptional and post-translational levels (Jurat-Fuentes and Jackson, 2012).

1.4.2 Virulence factors produced during the vegetative phase

Bt also synthesizes other insecticidal proteins that are expressed and secreted to the medium during the exponential phase of growth. There are two types of soluble proteins secreted during the vegetative phase: the vegetative insecticidal proteins (Vip proteins) (Crickmore, 2013; Estruch et al., 1996) and the secretable insecticidal proteins (Sip proteins) (Donovan et al., 2006). Vip proteins play an important role in the overall insecticidal activity displayed by Bt strains (Donovan et al., 2001) and have been considered a relevant alternative or complement to Cry and Cyt protoxins for insect pest control due to their different host range, toxicity and their unique mode of action (Jurat-Fuentes and Jackson, 2012). Currently, the *Bacillus thuringiensis* Toxin Nomenclature Committee (Crickmore, 2013) has identified and classified 4 different Vip protein families (Vip1, Vip2, Vip3 and Vip4) and a single Sip protoxin family. Vip1 and Vip2 are binary toxins with high insecticidal activity against some coleopteran (Gatehouse, 2008) and sap-sucking insect pest (Palma et al., 2014; Sattar and Maiti, 2011). Vip3 proteins are toxic against lepidopteran species (Estruch et al., 1996) while the toxicity host range of Vip4Aa1 still remains unknown. The Sip protoxin is the first member of a new class of Bt secreted proteins showing activity against coleopteran larvae (Donovan et al., 2006).

1.4.3 Other virulence factors

In addition to the proteins mentioned above, there are other virulence factors such as phospholipases, proteases, haemolysins, enterotoxins, β-exotoxins, metalloproteases (enhancins) and chitinases that are produced by some Bt strains. Some of them are synthesized by the vegetative cells in the insect midgut and facilitate the dissemination of bacteria through the peritrophic membrane and towards the hemocoel (Raymond et al., 2010; Schnepf et al., 1998).

β-exotoxins are secretable and heat-stable secondary metabolites with low molecular weight (700 Da). Because they are analogues to the nucleotide adenine, they act inhibiting DNA-dependant RNA polymerase (Gohar and Perchat, 2001), the β-exotoxins exhibit a wide host range including pollinators and other beneficial insects (Liu et al., 2010; Mac Innes and Bouwer, 2009; Raymond et al., 2010;

Schnepf et al., 1998) as well as many other species including mammals (McClintock et al., 1995). Therefore, the absence of β-exotoxins is an indispensable condition for any Bt strain before its registration to be use as a bio-pesticide.

Metalloproteases (enhancins) also contribute to Bt toxicity. The similarity with viral enhancin proteins is very low (around 20-30%) and they may act by degrading the mucin component of the peritrophic membrane (Fang et al., 2009). *Bacillus* enhancin-like (bel) protein has been reported to improve the toxicity of Cry1Ac against *Helicoverpa armigera* larvae (Fang et al., 2009).

1.4.4 Virulence factors from *Lysinibacillus* (*Bacillus*) *sphaericus*

Bin toxins from *Lysinibacillus* (*Bacillus*) *sphaericus* are formed by two different proteins: BinA and BinB. These proteins are highly conserved, co-transcribed from the same operon and accumulated as parasporal crystals during sporulation. They are found characteristically in highly toxic mosquitocidal strains (Baumann et al., 1988).

Mtx proteins are mosquitocidal toxins produced by *L. sphaericus* during vegetative growth (Carpusca et al., 2006; Park et al., 2010). These toxins seem to be soluble and do not produce crystalline inclusions. Mtx2 and Mtx3 are closely related and they exhibit an ETX/Mtx2 conserved domain. This domain is closely related to the *Clostridium* toxins and seems to be involved in the pore formation into the cell membrane (Berry, 2012; de Maagd et al., 2003).

2. THE δ-ENDOTOXINS OF Bt

2.1 Classification of Cry and Cyt proteins

The number of Bt genes that encode for new insecticidal proteins is continuously increasing and it requires a rational and useful nomenclature system. The first attempt to make a classification was through the use of Roman numerals, based on the activity of the crystal protein. E.g. CryI grouped those proteins toxic for Lepidoptera; CryII those proteins displaying activity against both Lepidoptera and Diptera; CryIII those proteins showing activity against Coleoptera; and CryIV those proteins exhibiting toxic activity mainly against species of Diptera (Höfte and Whiteley, 1989). Unfortunately, this classification system presented some drawbacks. On the one hand, the activity of the new toxin had to be assayed against a list of insects before the protein could be named and some of them were,

in fact, non-toxic. What is most confusing, some proteins showing a high identity and similarity were placed in the same taxonomic group; however, at least some of them, showed different host range (Crickmore et al., 1998). The *Bacillus thuringiensis* Toxin Nomenclature Committee was created to avoid the disadvantages of the previous classification system and a novel system was proposed (Crickmore, 2013; Crickmore et al., 1998). In this new system, the classification was developed on the basis of the identity and similarity of the aminoacidic sequence. So, a four-rank based system was assigned to novel toxins to determine the degree of pairwise similarity to the closest toxin. The first level of similarity was appointed by an Arabic number (e.g. Cry4), the second level by an uppercase letter (e.g. Cry4A), the third level by a lowercase letter (e.g. Cry4Aa), and the fourth level was also appointed by an Arabic number (e.g. Cry4Aa1). In this way, two proteins sharing less than 45% identity were assigned a different Arabic number (e.g. Cry4 and Cry11). Two proteins sharing an identity between 45 and 78% were assigned the same first Arabic number followed by a different capital letter (e.g. Cry4A and Cry4B); two proteins sharing greater than 78 and less than 95% identity were assigned the same first Arabic number, followed by the same capital letter and then by a different small letter (e.g. Cry4Aa and Cry4Ab); and finally, those proteins sharing more than 95% were differentiated by an additional Arabic number (e.g: Cry4Aa1 and Cry4Aa2) (Crickmore, 2013; Crickmore et al., 1998). This database, which is continuously updated, comprise nowadays 952 insecticidal Bt genes coding which have been classified in four different classes and a number of families (www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/*Bt*/). Into the Cry class, there are 78 Cry protein families (Cry1-Cry78) including more than 770 different Cry protoxins while in the Cyt class there are three Cyt protein families (Cyt1-Cyt3) containing a total of 38 Cyt protoxins. The insecticidal proteins secreted during the growing phase are taxonomically grouped into the Vip class, which currently comprise 138 different protoxins classified into 4 families (Vip1-Vip4), and the Sip1 class with only a member described so far (Sip1Aa1). The insecticidal proteins produced by *L. sphaericus,* such as Etx_Mtx2 proteins or Binary toxins, show considerable differences in their tertiary structure compared to the Bt insecticidal proteins described above, and therefore, according to the classification criteria, they do not belong to any of the previously described classes. In addition, the number of

unclassified insecticidal proteins in the current nomenclature systems, and the need to re-evaluate the nomenclature is becoming more and more a need (Chattopadhyay and Banerjee, 2018).

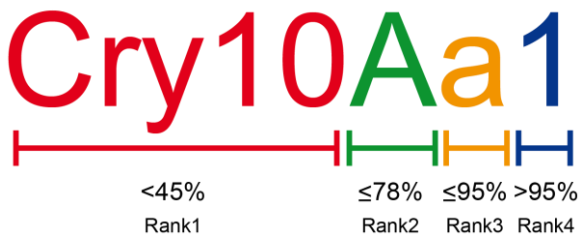


Figure 2. Nomenclature system adopted for insecticidal toxins produced by Bacillus thuringiensis. Four ranks are used to group up toxins according to their amino acid sequence identity. Primary, secondary and tertiary rank distinguish proteins with at least 45, between 45 and 78 and up to 95% sequence identities, respectively. Quaternary rank differentiates between alleles of the same gene found in different Bt isolates, although they may share the exact same sequence.

2.2 Structure of Cry and Cyt proteins

Based on its molecular structure and its homology, the largest group of Cry proteins is formed by the three-domain Cry proteins. Three-domain Cry toxins are divided into two main types, the large 130 kDa proteins and the truncated 65-70 kDa protoxins that lack the C-terminal region. The 130 kDa peptide is inactive and designated as a protoxin. The larval midgut proteases convert this protoxin into an active fragment through its proteolytic processing (Bravo et al., 2013; De Maagd et al., 1999). The active toxins possess the following three conserved domains. The N-terminal domain (Domain I) comprises a seven α-helix cluster that is responsible of toxin membrane insertion and pore formation. Furthermore, this cluster is subjected to proteolytic cleavage in all three-domain Cry proteins during toxin activation (Schnepf et al., 1998). The middle domain (Domain II) consists of three antiparallel β-sheets and plays an important role in toxin-receptor interactions (Jenkins and Dean, 2000). Lastly, the galactose-binding domain (Domain III) is a two antiparallel β-sheets sandwich that is also involved in receptor binding and pore formation. Active three-domain Cry toxins contain up to five typical conserved blocks in their sequence (Schnepf et al., 1998). These blocks are highly conserved and concentrated mainly in the center of the domains and at the junctions between them (Bravo et al.,

2013). Although this group of Cry proteins share a remarkably similar and conserved three-domain structure, they differ in their amino acid sequences (Bravo et al., 2007; de Maagd et al., 2001; de Maagd et al., 2003; Pardo-López et al., 2013). There are many other Cry proteins included in the no three-domain group, including Etx_Mtx2 proteins, binary toxins or parasporins.

In contrast to Cry proteins, Cyt proteins exhibit a general cytolytic (haemolytic) activity *in vitro* and dipteran specificity *in vivo* (Butko, 2003; de Maagd et al., 2003). Their three-dimensional structure shows that Cyt proteins are formed by a single domain with a β-sheet surrounded by two α-helical layers (Cohen et al., 2011, 2008). To date, the *Bacillus thuringinesis* Toxin Nomenclature Committee (Crickmore, 2013) has classified three different Cyt protein types (primary rank) with toxicity mostly against some mosquitoes and black flies (de Maagd et al., 2003; Soberón et al., 2013). However, there are other Cyt proteins with toxic activity against a different range of insects, including lepidopteran and coleopteran larvae (Guerchicoff et al., 2001).

2.3 Mode of action of Cry and Cyt proteins

The mode of action of Cry proteins involves several events that must be completed after the ingestion of the protoxin in order to lead to insect death. Following ingestion, the crystals are solubilized by the alkaline conditions in the midgut of the insects and are subsequently proteolytically converted into toxic fragments (de Maagd et al., 2003). During proteolytic activation, peptides from the N-terminus and C-terminus are cleaved from the full protein. Activated toxins are able to bind to specific receptors (glycoprotein or glycolipid) located on the apical microvillus membranes of epithelial midgut cells (Griffitts et al., 2005). After binding, the toxin adopts a conformational change allowing its oligomerization and its insertion into the cell membrane forming a pore. The formation of this pore or cation-selective channel leads to the lysis of the midgut insect cell by osmotic shock (Bravo et al., 2004). In addition to the toxin action, spores may pass through the channel, to colonize and germinate in the hemolymph and contribute to insect death by septicemia (Raymond et al., 2008).

Two different modes of action have been proposed for Cyt proteins (de Maagd et al., 2003; Soberón et al., 2013). One suggests a pore-formation model (Promdonkoy and Ellar, 2003, 2000) whereas the other supports a less specific detergent action mechanism (Butko, 2003; de Maagd et al., 2003;

Soberón et al., 2013). According to the former, Cyt binds as a monomer that then undergoes conformational changes. Its C-terminal half, composed mainly of β-strands, is inserted into the membrane and the N-terminal half, comprising mainly α-helices, is exposed on the outside of the membrane (Cohen et al., 2011; Rodriguez-Almazan et al., 2011). Oligomerization on the cell membrane forms β-barrel pores (Li et al., 1996; Rodriguez-Almazan et al., 2011), that induce equilibration of ions and net influx of water, cell swelling, and eventual colloid-osmotic lysis (Knowles and Dow, 1993; Knowles and Ellar, 1987). Consistent with a detergent-like mechanism, Cyt toxins are rather adsorbed onto the surface as aggregates thereby causing nonspecific defects in membrane lipid packing, through which intracellular molecules can leak by an all-or-nothing mechanism (Butko et al., 1997, 1996; Manceva et al., 2005).

Another important role of Cyt proteins is their ability to synergize with other Bt proteins, increasing the insecticidal damage of Cry toxins and also reducing the degree of insect resistance to certain Cry proteins (Soberón et al., 2013). Therefore, it is a fact the potential of these Cyt proteins in insect control (Soberón et al., 2013).

3. BT TOXINS ACTIVE AGAINST DIPTERA

To date, a number of Cry proteins have been reported to be toxic to a wide variety of insect species classified in the orders Lepidoptera, Coleoptera and Diptera (Salehi Jouzani et al., 2008), and more recently also in other insect orders as well as other non-insects organisms such as nematodes (Wei et al., 1993) and mites (Palma et al., 2014). Within Bt, there is a number of serovars (*israelensis*, *jegathesan*, *darmstadiensis*, *kyushensis*, *medellin*, *fukuokaensis*, *higo* etc.), each one of those includes a large number of Bt strains that contain genes that code for proteins with known insecticidal activity against an increasing number of dipteran species. An updated list of genes that express proteins with demonstrated insecticidal activity is as follows: *cry1*, *cry2*, *cry4*, *cry10*, *cry11*, *cry16*, *cry19*, *cry20*, *cry24*, *cry27*, *cry30*, *cry32*, *cry39*, *cry44*, *cry47*, *cry48*, *cry49*, *cry50*, *cry54*, *cry56*, *cry60*, *cyt1 and cyt2* (Table 2).

3.1 Cry toxins from Bt ser. *israelensis*

Bt ser. *israelensis* (Bti) was the first Bt serotype found to be toxic against dipteran larvae (Goldberg and Margalith, 1977). Bti is the most powerful and environmental-friendly biological alternative component in integrated programs to control disease vectors (Fillinger et al., 2003; Margalith and Ben-Dov, 2000). Bti is much more effective against many species of mosquito and black fly larvae than any previously known bio-control agent (Ben-Dov, 2014). The Bti crystal is composed by six Cry proteins (Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa and Cry60A/Cry60B) and two Cyt proteins (Cyt1Aa and Cyt2Ba).

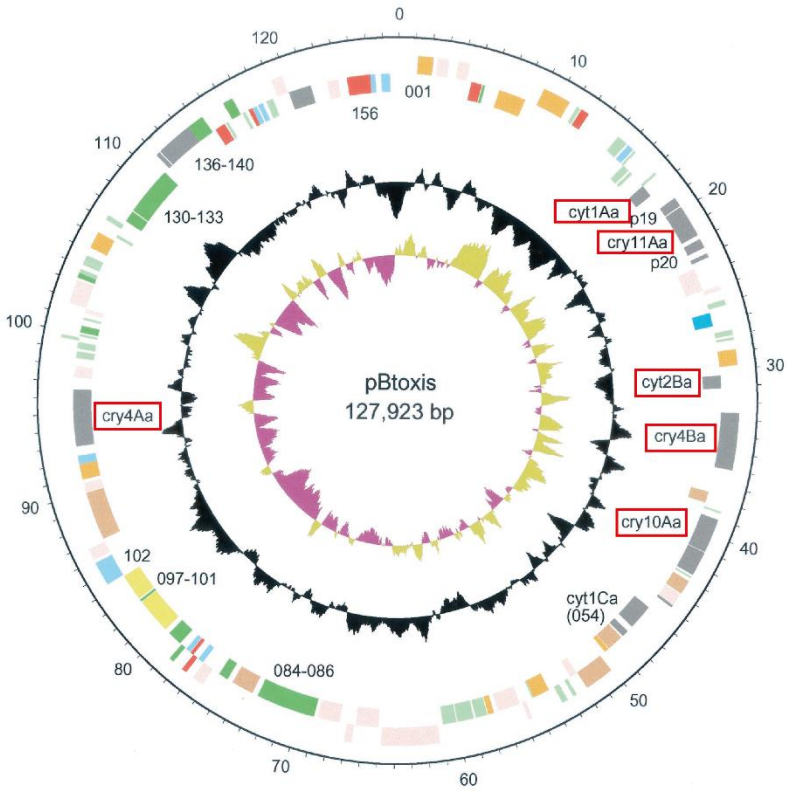


Figure 3. Schematic representation of the plasmid contained in Bti which harbours the *cry4Aa*, *cry4Ba*, *cry10Aa*, *cry11Aa*, *cyt1Aa* and *cyt2Ba* insecticidal genes. The parasporal cristal of Bti is mainly composed by Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa proteins with a minor representation of Cry10Aa and Cyt2Ba proteins. Image adapted from Berry et al., 2002.

Cry4 proteins

One of the crystal components of Bti is the Cry4Aa protein, formed by 1.180 amino acids and with a molecular mass of about 135 kDa. Another one is Cry4Ba with 1.141 amino acids and 128 kDa

approximately. These large protoxins form crystals spontaneously via inter- and intra-molecular disulphide bonds by their conserved C-terminal halves (Bietlot et al., 1990; Couche et al., 1987).

The Cry4A host range covers the following mosquito species: *A. aegypti, A. stephensi, A.gambiae, C. pipiens, and C. quinquefasciatus*. Several studies have provided evidence that the *Culex* species are the most susceptible to these proteins while the species of the *Anopheles* and *Aedes* genera are less susceptible (Angsuthanasombat et al., 1992; Bourgouin et al., 1988; Beltrão and Silva-Filha, 2007; Delecluse et al., 1993; Otieno-Ayayo et al., 2008; Sandrine Poncet et al., 1995).

Cry4B is another of the main proteins produced by Bti. This protein, showed high toxic activity against *Aedes aegypti* and *A. stephensi* larvae but it was totally inactive against larvae of species of the genus *Culex* (Abdullah et al., 2003; Angsuthanasombat et al., 1992; Delecluse et al., 1993; Poncet et al., 1995; Promdonkoy et al., 2005). The toxicity of Cry4B toxin toward *A. aegypti* and *A. stephensi* larvae was higher than that of the Cry4A (Delecluse et al., 1993). The putative loops 1 and 2 of domain II of the protein are responsible for its activity and mutations in putative loop 3 produce an increase in toxicity against Culex (Abdullah et al., 2003). Cry4Ba has also activity against *C. tepperi* (Hughes et al., 2005).

Cry10Aa protein

The Cry10A protein, whose primary structure consists of a 675 amino acid chain, is a minor component of the crystal produced by the Bt strains of serovar *israelensis* (Garduno et al., 1988; Lee et al., 1985; Thorne et al., 1986) which differs markedly from other major proteins such as Cry4Aa and Cry4Ba. However, it has been described that the 2025 bp *cry10A* gene (*orf1*) is followed by a second gene called *orf2*, which has a similarity of more than 60% with the caboxyl end of proteins Cry4Aa and Cry4Ba (Berry et al., 2002). That is the reason why some authors interpret that Cry10Aa and *orf2* could be a variant of the *cry4*-type genes. The *orf2* codes for a 56 kDa protein of molecular mass and, therefore, when the complete operon is cloned, two proteins of 68 (*orf1*) and 56 (*orf2*) kDa are expressed. Parasporal bodies formed by the complete Cry10Aa (Orf1-Orf2) are as active to *A. aegypti* as the Cry4 toxins (Hernández-Soto et al., 2009).

Cry11 proteins

The Cry11 family belongs to a large group of δ-endotoxins composed of three different domains and is composed of active proteins against some members of Diptera (Florez et al., 2018; Gutierrez et al., 2001). Cry11Aa from Bti is a 72 kDa protoxin that is located in a particular operon where the main gene (1941 bp) is flanked by two other small genes known as *p19* and *p20* (Dervyn et al., 1995; Wu and Federici, 1995). Among individual toxins from Bti Cry11A is the second most abundantly produced toxin, after Cyt1A. Cry11A proteins have a high toxicity against both *Aedes* and *Culex* genera while their insecticidal activity is lower against larvae of the *Anopheles* species. (Bukhari and Shakoori, 2009; Chang et al., 1992; Donovan et al., 1988; Poncet et al., 1995; Yamagiwa et al., 2004). This protein is activated in the insect midgut by proteolytic cleavage resulting in two fragments of 38 and 30 kDa with the capacity to bind the midgut microvilli (Dai and Gill, 1993; Beltrão and Silva-Filha, 2007; Revina et al., 2004).

In the case of *A. aegypti*, Cry11Aa may interact with different midgut brush border membrane receptors, a GPI anchored alkaline phosphatase (GPI-ALP) (Fernandez et al., 2006), the aminopeptidase N (Chen et al., 2009b) and the cadherin (Chen et al., 2009a). The protein binds also to Cyt1Aa as a membrane-bound receptor, increasing its activity (Pérez et al., 2005). In *A. albimanus* alfa-amylase has been described as a putative binding receptor for Cry11Aa (Fernandez-Luna et al., 2010). There are other midgut proteins, such as ATP binding protein, that increase the toxicity of this protein against *C. quinquefasciatus* third instar larvae (Zhang et al., 2017). Cry11Aa is also toxic against other dipterans such as *Chironomus tepperi* and *Tipula oleraceae* (Feldmann et al., 1995; Hughes et al., 2005).

There are other two proteins in Cry11 family phylogenetically related to Cry11Aa: Cry11Bb (94kDa) produced by Bt ser. *medellin* and Cry11Ba (81kDa) produced by Bt ser. *jegathesan*. These proteins share similar insect specificity and their activity is higher than that of Cry11A (Delecluse et al., 1995; Orduz et al., 1998). Three different *A. aegypti* midgut proteins, i.e., cadherin, AaeALP1, and AaeAPN1, are involved in Cry11Ba binding to *A. aegypti* midgut brush border membranes (Likitvivatanavong et al., 2011).

Cry60A/Cry60B proteins

In Bt ser. *jegathesan*, the *cry60A* (960 bp) and *cry60B* (912 bp) genes are forming an operon. These two ORFs have also been detected in Bt ser. *malayensis* 4AV1 (Sun 2013) and Bt ser. *israelensis* ATCC 35646 (Anderson et al., 2005). Interestingly, the operon that contains the cry60A (960 bp) and cry60B (912 bp) genes has exactly the same structure in these three Bt strains classified in three different serovars. Individual or joint expression of the cry60A and cry60B genes in a Bt strain (acrystalliferous) produces crystal components (33 and 35 kDa, respectively) that show moderate insecticidal activity against fourth instar *C. quinquefasciatus* larvae. Cry60Aa and Cry60Ba should not be referred to binary toxins because neither of them depends on the other to be expressed or exercise its insecticidal activity on the host insect (Sun et al., 2013).

3.2 Other toxins specific to Diptera

In addition to the Bt ser. *israelensis* toxins, there are some other proteins from different Bt serovars showing toxic activity against several species of Diptera. For example, Cry24Aa from Bt ser. *jegathesan*, showed toxic activity against *Culex quinquefasciatus* and *A. aegypti* (Kawalek, 1998). Cry24Ca also exhibited larvicidal activity against *A. aegypti* (Berón and Salerno, 2007). Cry44Aa from Bt ser. *entomocidus*, showed high toxic activity against *Culex pipiens* and *A. aegypti*, although the activity against *A. stephensi* was lower (Ito et al., 2006). Three other families with specific insecticidal activity are: Cry30 (Cry30Fa1 and Cry30Ga1), Cry32 (Cry32Ba1, Cry32Ca1 and Cry32Da1) and Cry56, all of them showing insecticidal activity against *A. aegypti* larvae (Tan et al., 2010; J. Zhu et al., 2010; Jun Zhu et al., 2010). Within the Cry27 family, it has been reported that the Cry27A protein produced by a strain of Bt ser. *higo* shows activity against *A. stephensi* species but is not toxic to species classified in the genus *Culex* and *Aedes* (Saitoh et al., 2000). Another protein of the Cry39 family, Cry39A, has also been found to be highly toxic against this mosquito larvae (Ito et al., 2006, 2002; Saitoh et al., 2000). Finally, another protein of the Cry47 family, Cry47Aa, has also been described as active against dipteran species, such as the sheep blowfly *Lucilia cuprina* (Gough et al., 2005; Kongsuwan et al., 2005).

3.3 Anti-dipterous toxins with cross-activity

Cry1 protein

Cry1 family is typically active to species of the lepidopteran order (Chilcott and Wigley, 1994). However, several proteins belonging to this family also display insecticidal activity against species of the dipteran order. For example, Cry1Ab7 protein, was active against *A. aegypti* larvae (Haider et al., 1987) and Cry1Ca showed toxic activity against larvae of different mosquito species such as *A. aegypti*, *C. quinquefasciatus* and *A. gambiae* (Abdul-Rauf and Ellar, 1999; Smith et al., 1996). Other Cry1 proteins show activity against different species of flies. Cry1Ac, for example, was active against *Glossina mortisans* adults (Diptera; Glossinidae) (Omolo et al., 1997) and Cry1Ba had toxicity against *Musca domestica* larvae (Diptera; Muscidae) (Zhong et al., 2000) and also *Lucilia cuprina* larvae (Diptera; Calliphoridae), when it was applied in high concentrations (Heath et al., 2004).

Cry2A protein

Within the Cry2 family, it has been described that the Cry2A protein has a wide activity that includes species of the orders Lepidoptera and Diptera (Ahmad et al., 1989; Saleem and Shakoori, 2010). Cry2Aa, it is toxic against the dipteran order, being its activity demonstrated mostly using *A. aegypti* (Misra et al., 2002; Nicholls et al., 1989; Park et al., 1999; Widner and Whiteley, 1989), but there are other assays with *C. quinquefasciatus*, *Culex fatigans* and *A. stephensi* (Misra et al., 2002; Moar et al., 1994). Cry2Ag has also been reported as a toxic protein for the larvae of *A. aegypti* (Liang et al., 2011), while Cry2Ab25 has showed high mortality against *Rhagoletis cerasi* larvae (Diptera; Tephritidae) (Sevim et al., 2012).

Other proteins

There are a number of less studied proteins which are simultaneously active against larvae of several dipteran and lepidopteran species. This is the case of Cry19A identified in a Bt ser. *jegathesan* strain that exhibited toxic activity against *C. pipiens* and *A. stephensi* (Rosso and Delécluse, 1997). Cry19B, a close member of this family derived from Bt serovar *higo*, showed activity against *Culex molestus* larvae, but not against *A. stephensi* (Hwang et al., 1998). Cry20Aa is another mosquitocidal protein produced by a strain of Bt ser. *fukuokaensis* that has been shown to be toxic to larvae of *A. aegypti*

and *C. quinquefasciatus*. Nevertheless, the toxicity was not high perhaps due to the rapid degradation of the protein (Lee and Gill, 1997). Moreover, Cry54 protein showed toxic activity against *A. aegypti* larvae (Tan et al., 2009). Finally, Cry50Ba has been described as highly active against *C. quinquefasciatus* (Zhang et al 2017).

3.4 Anti-dipterous toxins from other microorganism

Mtx from *L. sphaericus*

The bacterium *L. sphaericus* produces some mosquitocidal factors such as Mtx proteins that display toxicity against *C. quinquefasciatus*. Mtx1 protein was first identified in the SSII-1 strain (Thanabalu et al., 1991) and was highly active against *C. quinquefasciatus*. The Mtx2 protein was also identified in SSII-1 *L. sphaericus* (Thanabalu and Porter, 1996) and is unrelated to Mtx1 protein (Liu et al., 1996; Thanabalu and Porter, 1996; Wirth et al., 2001). When Mtx1 and Mtx2 were assayed independently against *C. quinquefasciatus* (Wirth et al., 2014), the first of these two toxins was comparatively more toxic than the second. Activity of the Mtx2 toxin towards the two mosquito species is different for natural variants of the toxin, and the amino acid residue 224 has been shown to be critical in determining the optimal target; threonine favours activity against *A. aegypti*, whereas lysine favours activity against *C. quinquefasciatus* (Chan et al., 1996). Some results suggest that the modes of action of both Mtx1 and Mtx2 share some common features with those of Cry toxins in mosquitoes (Wirth et al., 2014). There is another *mtx* gene called *mtx3* from *L. sphaericus* SSII-1 with homology to the Mtx2 mosquitocidal toxin. The *mtx3* gene is highly conserved and widely distributed in both high- and low-toxicity mosquitocidal strains of *L. sphaericus* (Liu et al., 1996). Lastly, *mtx4* is a pseudogene member for the Mtx2 family toxins, but the activity of the Mtx4 putative toxin has not been assessed (Berry, 2012).

Cry48/Cry49 from *L. sphaericus*

Cry48Aa is a three-domain Cry toxin, and is closely related to the Cry4 toxins, while Cry49Aa is a member of the mosquitocidal Bin family of *L. sphaericus*. Neither Cry48Aa nor Cry49Aa were toxic when assayed individually to *C. quinquefasciatus*, but when the proteins were co-administered at the

optimum 1:1 ratio, high levels of toxicity against this mosquito species were observed (Jones et al., 2007, 2008).

Cry16 from *Clostridium bifermentans*

Toxins from *Clostridium bifermentans* subsp. *malaysia* are highly toxic to *Anopheles* species, however its toxicity against *C. pipiens* or *A. aegypti* is 10 times lower than that of Bti (Thiery et al., 1997). Some authors cloned and expressed the 71 kDa protein, named Cry16A, from *C. bifermentans* and its mosquitocidal activity was demonstrated (Barloy et al., 1996). This was the first-described protein produced by an anaerobic bacteria showing toxicity against mosquito larvae (Barloy et al., 1996; Thiery et al., 1997). The protein has similar amino acid sequence compared to Cry1, Cry3 and Cry4 toxins (Höfte and Whiteley, 1989). This correspondence is stronger within the amino-terminal domain, especially in blocks I to IV. The highest similarity occurs with Cry3 proteins, which lack also the C-terminal region (Lereclus et al., 1993). Cry16 could belong to a novel class of mosquitocidal toxins, due to the absence of cross-reaction with antibodies raised against Bti or *L. sphaericus* (Nicolas et al., 1993).

3.5 Anti-dipterous Cyt Toxins

Cyt1 protein

The proteins of the Cyt1 family do not bear any similarity with any of the Cry families (Cry1-Cry78) currently described (Crickmore et al., 1998). Of all the proteins described in the Cyt1 family, the Cyt1A protein has undoubtedly been the most widely studied. Cyt1A adopts a typical cytolysin fold containing a ß-sheet held by two surrounding alfa-helical layers (Cohen et al., 2011). The insecticidal activity of Cyt1A for the larvae of several dipteran species has been reported by several authors (Chilcott and Ellar, 1988; Otieno-Ayayo et al., 2008; Waalwijk et al., 1992; Wu et al., 1994). Efficient expression of the Cyt1A (molecular mass of 27 kDa) protein requires the presence of a 20 kDa “helper” polypeptide (Adams et al., 1989). Proteolytic digestion of Cyt1A protein produces fragments of 22-25 kDa that are more effective than the native protoxin *in vitro* (Al-yahyaee and Ellar, 1995). This toxin shows haemolytic and cytolytic *in vitro* activity to cells of vertebrates and invertebrates (Bourgouin et al., 1988; Gill et al., 1987; Ward et al., 1986), apparently due to the interaction between its hydrophobic

segment and membrane phospholipids from the midgut epithelial cells (Butko et al., 1997, 1996; Haider and Ellar, 1989; Knowles et al., 1989). Cyt1Aa has been tested as a full-length soluble protein mixed with diet against a number of species of the Brachycera suborder and has been found to be toxic against first-instar *Lucilia sericata* (Diptera; Calliphoridae), *Lucilia cuprina* (Diptera; Calliphoridae) and *Calliphora stygia* (Diptera; Calliphoridae). Furthermore, when Cyt1Aa was previously digested with trypsin, the toxicity was four to six times higher. However, Cyt1Aa purified crystals were not toxic (Chilcott et al., 1998). Cyt1A showed also toxic activity against *T. paludosa* (Oestergaard et al 2007).

To date, no highly toxic Bt toxins have been found against *Ceratitis capitata* (Diptera; Tephritidae) in the field. However some authors showed that previously solubilized protoxin of Cyt1A showed, under controlled laboratory conditions, a moderate toxicity against *C. capitata* larvae (Vidal-Quist et al., 2010). Apparently Bti crystals are not efficiently solubilized below pH 9 and the pH of *C. capitata* third instar larvae and adult midgut has been calculated as 8 and 7.5, respectively (Orduz et al., 1996).

Cyt1Ab1, a protein that has a similarity of 86% with Cyt1Aa1, is also active, although to a lesser extent than Cyt1Aa1, against *Aedes*, *Anopheles* and *Culex* (Thiery et al., 1997). The ability of Cyt1B to induce mortality and reduce the damage caused by of *Liriomyza trifoli* (Diptera; Agromyciidae) mining larvae also have been described (Payne et al., 1995).

Cyt2 protein

The proteins of the Cyt2 family have been identified and characterized in several Bt subspecies: Cyt2Aa from Bt ser. *kyushensis* (Koni and Ellar, 1994) and *darmstadiensis* (Promdonkoy et al., 2003), Cyt2Ba from Bti (Guerchicoff et al., 1997), Cyt2Bb from Bt ser. *jegathesan* (Cheong et al 1997) and Cyt2Bc from Bt ser. *medellin* (Juárez-Pérez et al., 2002). There is a correlation between the Bt activity against dipteran species and the presence of Cyt2 proteins (Guerchicoff et al., 2001).

Cyt2Aa1 from Bt ser. *kyushensis* displays low identity (39%) with Cyt1Aa from Bti, but the structural homology is 70% (Guerchicoff et al., 1997). Moreover, both are processed in similar domains (Koni and Ellar, 1994) probably because they share a high degree of structural similarity (Koni and Ellar,

1994; Li et al., 1996). Cyt2Aa1 is a 29.2 KDa (259 amino acids) protein and its crystal structure is resolved. It consists on a single α-β domain comprising two outer layers of α-helix hairpins and a β-sheet in between (Li et al., 1996). The protein does not show haemolytic activity as a protoxin, however, N and C terminal segments are cleaved by proteolysis leading to dimer dissociation and toxin activation. Cyt2Aa1 showed LC_{50} values in a range of 0.5 and 4 µg/ml against *Culex*, *Anopheles* and *Aedes* (Koni and Ellar, 1994). Furthermore, Cyt2Aa2 is produced by Bt ser. *darmstadiensis* and exhibited moderate activity against *Culex* and *Aedes* larvae. It showed haemolytic activity against sheep erythrocytes too (Promdonkoy et al., 2003). Moreover, Cyt2Aa3, from the Bt strain MC28, exhibited toxic activity against *C. tepperi* and *C. quinquefasciatus* larvae (Yu et al., 2012).

The 263 amino acid Cyt2Ba protein (30.1 kDa) from Bti shows an identity percentage of 41% with Cyt1Aa and 67% with Cyt2Aa1 (Guerchicoff et al., 1997). It was less active than Cyt1Aa against *A. aegypti*, *C. pipiens*, *C. quinquefasciatus* and *A. stephensi* larvae (Juárez-Pérez et al., 2002; Wirth et al., 2001). Moreover, solubilisation or trypsin activation was mandatory for its haemolytic activity (Juárez-Pérez et al., 2002). The crystal structure of the proteolytically cleaved active form of Cyt2Ba has been described. The structure resembles that of the protoxin form of Cyt2Aa and also the fungal volvatoxin A2 (Cohen et al., 2008). Cyt2Bb, from Bt ser. *jegathesan*, is a 263 amino acids protein with 30,1 kDa and displayed mosquitocidal activity against *A. aegypti* larvae. The toxicity was lower than the one of Cyt1Aa, however they shared similar haemolytic activity (Cheong and Gill, 1997). Cyt2Bc from Bt ser. *medellin* is a 29,7 kDa toxin (260 amino acids) that showed mosquitocidal activities against *A. aegypti, A. stephensi, C. pipiens and C. quinquefasciatus*. However the toxicity was lower than Cyt1Aa and Cyt2Ba. Trypsin treatment was needed for its haemolytic activity (Juárez-Pérez et al., 2002).

3.6 Toxins with synergistic activity against Diptera

The high toxicity of the complete crystal compared to what would be expected, in the event of an additive effect of the toxicity of each of the proteins that compose it, has been attributed to the synergistic activity that occurs between some of the proteins that make up the crystal. The synergistic activity between proteins has been studied in more detail in Bti than in any other Bt serovar. Some of

the combinations for which a synergistic effect has been described are the following: Cry4Aa+Cry4Ba, Cry11Aa+Cry4Aa, Cry11Aa+Cry4Aa+Cry4Ba. All these combinations of proteins interact synergistically for a large number of species classified in the genera: *Aedes*, *Anopheles* and *Culex* (Angsuthanasombat et al., 1992; Crickmore et al., 1995; Delecluse et al., 1993; Poncet et al., 1995; Ricoldi et al., 2018; Soberón et al., 2013). Moreover, Cry4Ba had synergistic effect with Cry10Aa against *C. pipiens* (Delécluse et al., 1988) and with Cry11Aa against *A. aegypti* and *A. albimanus* larvae (Crickmore et al., 1995; Fernandez-Luna et al., 2010). Although Cyt1A is the least toxic, it is the strongest synergist among the whole δ-endotoxins against *A. aegypti* (Crickmore et al., 1995; Hernández-Soto et al., 2009; Wu et al., 1994). Cyt1A interacts synergistically with Cry11A against *C. quinquefasciatus* (Chang et al., 1993) and *A. albimanus* (Fernandez-Luna et al., 2010), and with Cry4Ba against *A. albimanus* (Fernandez-Luna et al., 2010). In addition to significantly reducing the lethal concentration, the synergistic effect of Cyt1A also plays an important role retarding the appearance of resistance to Cry proteins in the case of *C. quinquefasciatus* (Georghiou and Wirth, 1997; Wirth et al., 2005; Wirth and Georghiou, 1997). Furthermore, Cyt2Ba, that is present in very low quantities in Bti crystals, has some synergistic effect with Cry4A against *A. aegypti* (Manasherob et al., 2006). Another Cyt protein from Bt ser. *darmstadiensis*, Cyt2Aa2, has highly synergistic activity with Cry4Ba from Bti against *A. aegypti* and *C. quinquefasciatus* larvae. It seems that Cyt2Aa2 not only shows a synergistic effect with Cry4Ba, which is active against *A. aegypti*, but also promotes the toxic activity of this Cry protein against *C. quinquefasciatus* larvae (Promdonkoy et al., 2005).

The synergy mechanism could be explained because Cyt1A protein can function as a membrane-bound receptor for Cry4Ba and Cry11A (Cantón et al., 2011; Elleuch et al., 2015; Pérez et al., 2005; Thomas and Ellar, 1983). In relation to Cry11Aa protein, Cyt1Aa inserts its β-sheet into the membrane and two of its components (loop β6-αE and part of β7) bind with high affinity to Cry11Aa, which subsequently is inserted into the larval epithelial membranes. Cyt1Aa seems to facilitate the formation of a pre-pore oligomeric structure that is able to form pores in synthetic lipid membrane vesicles (Pérez et al., 2005, 2007). However, oligomerization and membrane insertion of Cyt1A are dispensable for its synergistic activity (López-Diaz et al., 2013).

Cry toxins can also synergize with other proteins that are not in ser. *israelensis*, e.g. Cry11Ba from Bt ser. *jegathesan* presents synergistic activity in combination with Cry4Aa from Bti against *C. pipiens* larvae (Hayakawa et al., 2017). Furthermore, synergies have been described between Cyt proteins and *L. sphaericus* bacterium. In fact, Cyt1Aa, Cyt1Ab and Cyt2Ba combined with *L. sphaericus* binary toxins show an increase in toxic activity against *A. aegypti* and *C. quinquefasciatus* (Thiéry et al., 1998; Wirth et al., 2014, 2001, 2000). Moreover, Cyt1Aa is also capable of suppress resistance to *L. sphaericus* toxins in *C. quinquefasciatus* (Wirth et al., 2001, 2000). Also, Mtx1 and Mtx2 proteins from *L. sphaericus* are found to interact synergistically with a variety of Cry-toxins from Bti against *C. quinquefasciatus*.

Summarizing all these data, the synergistic mechanism between different kinds of toxins of Bt or *L. sphaericus* is an excellent strategy to increase the virulence of these microorganisms against relevant dipteran species.

Table 2. Insecticidal activities of Bt toxic factors against dipteran pest previously described in the literature.

Protein		Insect		Activity range LC50 (µg/ml)	References
Family	**Current Name**	**Family**	**Species**		
Cry1	Cry1Ab7	Culicidae	*Aedes aegypti*	NA	(Haider et al., 1987)
	Cry1Ac8	Glossinidae	*Glosina mortisans*	0.425-0.740	(Omolo et al., 1997)
	Cry1Ba1	Muscidae	*Musca domestica*	20	(Zhong et al., 2000)
		Calliphoridae	*Lucilia cuprina*	NA	(Heath et al., 2004; Johnson et al., 1998)
	Cry1Bc1	Muscidae	*Musca domestica*	79.4	(Johnson et al., 1998)
		Calliphoridae	*Lucilia cuprina*	308	(Johnson *et al.*, 1998)
			Chrysomya albiceps	807	(Johnson *et al.*, 1998)
	Cry1Ca1	Culicidae	*Aedes aegypti*	39.3-141	(Abdul-Rauf and Ellar, 1999; Smith et al., 1996)
			Anopheles gambiae	143-283	(Smith *et al.*, 1996)
			Culex quinquefasciatus	126	(Smith *et al*., 1996)
Cry2	Cry2Aa1	Culicidae	*Aedes aegypti*	37.06-79.46	(Donovan et al., 1988; Park et al., 1999; Ricoldi et al., 2018; Sims, 1997)
			Anopheles quadrumaculatus	0.37	(Sims, 1997)
			Anopheles triseriatus	2.84	(Sims, 1997)
			Culex quinquefasciatus	0.528	(L. Zhang et al., 2017)
	Cry2Aa2	Culicidae	*Culex quinquefasciatus*	1.63	(Moar et al., 1994)
	Cry2Aa4	Culicidae	*Aedes aegypti*	NA	(Misra et al., 2002)
			Anopheles stephensi	NA	(Misra *et al.*, 2002)
			Culex fatigans	NA	(Misra *et al*., 2002)
	Cry2Aa14	Culicidae	*Culex quinquefasciatus*	0.894	(Hire et al., 2009)
	Cry2Ab1	Culicidae	*Aedes aegypti*	23.42-35.80	(Ahmad et al., 1989; Liang et al., 2011; Ricoldi et al., 2018; Widner and Whiteley, 1989)
	Cry2Ab25	Tephritidae	*Ragoletis cerasi*	NA	(Sevim et al., 2012)
	Cry2Ag	Culicidae	*Aedes aegypti*	2.541	(Liang et al., 2011)
Cry4	Cry4Aa1	Culicidae	*Aedes aegypti*	0.264-13	(Abdullah et al., 2003; Angsuthanasombat et al., 1992; Angusthanasombat et al., 1991; Beltrão and Silva-Filha, 2007; Bourgouin et al., 1988; Crickmore et al., 1995; Delecluse et al., 1993; Ito et al., 2006b; Poncet et al., 1995; Ward et al., 1986)
			Anopheles gambiae	1.07-1.17	(Angsuthanasombat et al., 1992)
			Anopheles stephensi	0.521-7.4	(Delecluse et al., 1993; Delécluse et al., 1988; Ito et al., 2006b; Poncet et al., 1995)
			Culex pipiens	0.251-0.4	(Abdullah et al., 2003; Bourgouin et al., 1988;

Protein		Insect		Activity range LC50 (µg/ml)	References
Family	**Current Name**	**Family**	**Species**		
					Delecluse et al., 1993; Ito et al., 2006b; Poncet et al., 1995)
			Culex quinquefasciatus	0.052-5.04	(Abdullah et al., 2003; Angsuthanasombat et al., 1992; Wirth et al., 2014; L. Zhang et al., 2017)
	Cry4Ba1	Chironomidae	*Chironomus tepperi*	0.94	(Hughes et al., 2005)
		Culicidae	*Aedes aegypti*	0.12-0.94	(Abdullah et al., 2003; Angsuthanasombat et al., 1992; Beltrão and Silva-Filha, 2007; Crickmore et al., 1995; Delecluse et al., 1993; Delécluse et al., 1988; Poncet et al., 1995; Promdonkoy et al., 2005)
			Anopheles albimanus	1.3	(Fernandez-Luna et al., 2010)
			Anopheles gambiae	0.79	(Angsuthanasombat et al., 1992)
			Anopheles quadrumaculatus	0.25	(Abdullah et al., 2003)
			Anopheles stephensi	0.55-17	(Delecluse et al., 1993; Delécluse et al., 1988; Poncet et al., 1995)
			Culex quinquefasciatus	24.5	(Angsuthanasombat et al., 1992)
			Culex pipiens	NA	(Delécluse et al., 1988)
		Tipulidae	*Tipula oleraceae*	NA	(Waalwijk et al., 1992)
	Cry4Ba2	Culicidae	*Aedes aegypti*	NA	(Angsuthanasombat and Panyim, 1989)
	Cry4Cb1	Culicidae	*Aedes aegypti*	0.083	(J Zhu et al., 2010)
Cry10	Cry10Aa	Culicidae	*Aedes aegypti*	2.39-20.61	(Hernández-Soto et al., 2009)
Cry11	Cry11Aa 1	Chironomidae	*Chironomus tepperi*	0.56	(Hughes et al., 2005)
		Culicidae	*Aedes aegypti*	0.01-1.35	(Beltrão and Silva-Filha, 2007; Crickmore et al., 1995a; Delecluse et al., 1995; Donovan et al., 1988; Florez et al., 2018; Orduz et al., 1998a; Park et al., 1999; Poncet et al., 1995; Revina et al., 2004; Wu et al., 1994b)
			Anopheles albimanus	0.9	(Fernandez-Luna et al., 2010)
			Anopheles stephensi	0.135-0.455	(Bukhari and Shakoori, 2009; Delecluse et al., 1995; Feldmann et al., 1995; Ito et al., 2006b; Revina et al., 2004)
			Anopheles albimanus	6.759	(Orduz et al., 1998a)
			Culex pipiens	0.0086-0.268	(Delecluse et al., 1995; Revina et al., 2004)
			Culex quinquefasciatus	0.0138-0.133	(Chang et al., 1992; Cheong et al., 1997;

Protein		Insect		Activity range LC50 (µg/ml)	References
Family	**Current Name**	**Family**	**Species**		
					Florez et al., 2018; Orduz et al., 1998a; Wirth et al., 2014; L. Zhang et al., 2017)
		Tipullidae	*Tipula oleraceae*	NA	(Feldmann et al., 1995)
	Cry11Ba1	Culicidae	*Aedes aegypti*	0.0188-0.0236	(Delecluse et al., 1995; Orduz et al., 1998a)
			Anopheles albimanus	0.1056	(Orduz et al., 1998a)
			Anopheles stephensi	0.0427	(Delecluse et al., 1995)
			Culex pipiens	0,0101	(Delecluse et al., 1995)
			Culex quinquefasciatus	0.00653-0.0193	(Cheong et al., 1997; Orduz et al., 1998a)
	Cry11Bb1	Culicidae	*Aedes aegypti*	0.0179-0.846	(Florez et al., 2018; Juárez-pérez et al., 2003; Orduz et al., 1998a; Restrepo et al., 1997)
			Anopheles albimanus	0.166	(Orduz et al., 1998a)
			Anopheles stephensi	0.067	(Juárez-pérez et al., 2003)
			Culex pipiens	0.044	(Juárez-pérez et al., 2003)
			Culex quinquefasciatus	0.013-0.132	(Florez et al., 2018; Orduz et al., 1998a; Restrepo et al., 1997)
Cry16	Cry16	Culicidae	*Aedes aegypti*	185	(Barloy et al., 1996)
			Anopheles stephensi	129	(Barloy et al., 1996)
			Culex pipiens	156	(Barloy et al., 1996)
Cry19	Cry19Aa	Culicidae	*Anopheles stephensi*	1.039-24.926	(Rosso and Delécluse, 1997)
			Culex pipiens	0.18705-10.282	(Rosso and Delécluse, 1997)
	Cry19B		*Culex pipiens molestus*	5.93	(Hwang et al., 1998)
Cry20	Cry20Aa1	Culicidae	*Aedes aegypti*	648	(H. Lee and Gill, 1997)
			Culex quinquefasciatus	700	(H. Lee and Gill, 1997)
Cry24	Cry24Aa1	Culicidae	*Aedes aegypti*	189	(Kawalek, 1998)
			Culex quinquefasciatus	180	(Kawalek, 1998)
	Cry24Ca1	Culicidae	*Aedes aegypti*	0.476	(Berón and Salerno, 2007)
Cry27	Cry27Aa1	Culicidae	*Anopheles stephensi*	9.3	(Saitoh et al., 2000)
Cry30	Cry30Fa1	Culicidae	*Aedes aegypti*	0.00647	(Tan et al., 2010)
	Cry30Ga1	Culicidae	*Aedes aegypti*	7.101	(J Zhu et al., 2010)
Cry32	Cry32Ba1	Culicidae	*Aedes aegypti*	NA	(Takebe et al., 2001)
	Cry32Ca1	Culicidae	*Aedes aegypti*	NA	(Takebe et al., 2001)
	Cry32Da1	Culicidae	*Aedes aegypti*	NA	(Takebe et al., 2001)
Cry39	Cry39Aa1	Culicidae	*Anopheles stephensi*	0.75	(Ito et al., 2006a)

Protein		Insect		Activity range LC50 (µg/ml)	References
Family	**Current Name**	**Family**	**Species**		
Cry44	Cry44Aa1	Culicidae	*Aedes aegypti*	0.012	(Ito et al., 2006b)
			Anopheles stephensi	1.265	(Ito et al., 2006b)
			Culex pipiens	0.006	(Ito et al., 2006b)
Cry47	Cry47Aa1	Calliphoridae	*Lucilia cuprina*	NA	(Gough et al., 2005; Kongsuwan et al., 2005)
Cry50	Cry50Ba	Culicidae	*Culex quinquefasciatus*	0.074	(W. Zhang et al., 2017)
Cry54	Cry54Aa1	Culicidae	*Aedes aegypti*	9.02	(Tan et al., 2009)
Cry56	Cry56Aa1	Culicidae	*Aedes aegypti*	0.151	(J. Zhu et al., 2010)
Cry60	Cry60Aa	Culicidae	*Culex quinquefasciatus*	7.9	(Sun et al., 2013)
	Cry60Ba	Culicidae	*Culex quinquefasciatus*	5.5	(Sun et al., 2013)
	Cry60Aa+Cry60Ba	Culicidae	*Culex quinquefasciatus*	2.9	(Sun et al., 2013)
Cyt1	Cyt1Aa1	Calliphoridae	*Calliphora stygia*	27-305	(Chilcott and Ellar, 1988)
			Lucilia cuprina	33-296	(Chilcott and Ellar, 1988)
			Lucilia sericata	32-236	(Chilcott and Ellar, 1988)
		Chironomidae	*Chironomus tepperi*	31	(Hughes et al., 2005)
		Culicidae	*Aedes aegypti*	0.146-1.86	(Cheong and Gill, 1997; Hernández-Soto et al., 2009; Juárez-Pérez et al., 2002; Thiery et al., 1997; Torres-Quintero et al., 2018; Wirth et al., 2000)
			Anopheles stephensi	2.7-6.3	(Juárez-Pérez et al., 2002; Thiery et al., 1997)
			Culex pipiens	0.6-1.2	(Juárez-Pérez et al., 2002; Thiery et al., 1997)
			Culex quinquefasciatus	0.0004	(Juárez-Pérez et al., 2002)
		Tephritidae	*Ceratitis capitata*	NA	(Vidal-Quist et al., 2010)
		Tipullidae	*Tipula paludosa*	NA	(Oestergaard et al., 2007)
	Cyt1Aa2	Culicidae	*Aedes aegypti*	0.12-1.209	(Crickmore et al., 1995; Koni and Ellar, 1994; Ward et al., 1986)
			Anopheles gambiae	1-2	(Koni and Ellar, 1994)
			Culex pipiens	0.5-2	(Koni and Ellar, 1994)
	Cyt1Aa4	Culicidae	*Aedes aegypti*	0.06	(Wu et al., 1994b)
			Culex quinquefasciatus	>10	(Chang et al., 1993)
	Cyt1Ab1	Culicidae	*Aedes aegypti*	32.6-59	(Thiery et al., 1997; Wirth et al., 2001)
			Anopheles stephensi	20	(Thiery et al., 1997)
			Culex pipiens	5.7	(Thiery et al., 1997)
			Culex quinquefasciatus	32.9-114.5	(Wirth et al., 2001)
	Cyt1Ba1	Agromyciidae	*Liriomyza trifolii*	NA	(Payne et al., 1995)

Protein		Insect		Activity range LC50 (µg/ml)	References
Family	**Current Name**	**Family**	**Species**		
Cyt2	Cyt2Aa1	Culicidae	*Aedes aegypti*	1-4	(Koni and Ellar, 1994)
			Anopheles gambiae	1-2	(Koni and Ellar, 1994)
			Culex pipiens	0.5-4	(Koni and Ellar, 1994)
	Cyt2Aa2	Culicidae	*Aedes aegypti*	0.35-0.5	(Promdonkoy et al., 2005, 2003)
			Culex quinquefasciatus	0.25-0.5	(Promdonkoy et al., 2005, 2003)
	Cyt2Aa3	Chironomidae	*Chironomus tepperi*	36	(Yu et al., 2012)
		Culicidae	*Culex quinquefasciatus*	0.53	(Yu et al., 2012)
	Cyt2Ba1	Culicidae	*Aedes aegypti*	3.6-33	(Juárez-Pérez et al., 2002; Nisnevitch et al., 2006; Wirth et al., 2001)
			Anopheles stephensi	5.5	(Juárez-Pérez et al., 2002)
			Culex pipiens	5	(Juárez-Pérez et al., 2002)
			Culex quinquefasciatus	1.8-31.5	(Juárez-Pérez et al., 2002; Wirth et al., 2001)
	Cyt2Bb1	Culicidae	*Aedes aegypti*	6.8	(Cheong and Gill, 1997)
	Cyt2Bc1	Culicidae	*Aedes aegypti*	7	(Juárez-Pérez et al., 2002)
			Anopheles stephensi	11	(Juárez-Pérez et al., 2002)
			Culex pipiens	7.3	(Juárez-Pérez et al., 2002)
			Culex quinquefasciatus	1.8	(Juárez-Pérez et al., 2002)
Mtx1	Mtx1	Culicidae	*Aedes aegypti*	0.050	(Thanabalu et al., 1992)
		Culicidae	*Culex quinquefasciatus*	0.015	(Thanabalu et al., 1992)
		Culicidae	*Chironomus riparus*	4.06	(Partridge and Berry, 2002)
Mtx2	Mtx2	Culicidae	*Culex quinquefasciatus*	4.13-107	(Wirth et al., 2014)
	Mtx2 (SSII-1)	Culicidae	*Aedes aegypti*	14.5	(Chan et al., 1996)
			Culex quinquefasciatus	0.93	(Chan et al., 1996)
	Mtx2 (31-2)	Culicidae	*Aedes aegypti*	3.91	(Chan et al., 1996)
			Culex quinquefasciatus	3.90	(Chan et al., 1996)
Mtx3	Mtx3	Culicidae	*Aedes aegypti*	NA	(Liu et al 1996)
			Culex quinquefasciatus	NA	(Liu et al 1996)
Two part Toxins	Cry48Aa/Cry49Aa	Culicidae	*Culex quinquefasciatus*	0.0159-0.0063	(Jones et al., 2007)
Synergy	Cry2Aa/Cry2Ab	Culicidae	*Aedes aegypti*	51.3	(Ricoldi et al., 2018)
	Cry2Aa/Cry50Ba	Culicidae	*Culex quinquefasciatus*	0.0469	(W. Zhang et al., 2017)
	Cry4Aa/Cry4Ba	Culicidae	*Aedes aegypti*	0.052	(Poncet et al., 1995)
			Anopheles stephensi	0.016	(Poncet et al., 1995)
			Culex pipiens	0.036	(Poncet et al., 1995)
			Culex quinquefasciatus	1.49-315	(Wirth et al., 2014)
	Cry4Aa/Cyt1Aa	Culicidae	*Aedes aegypti*	0.075	(Crickmore et al., 1995)

Protein		Insect		Activity range LC50 (µg/ml)	References
Family	Current Name	Family	Species		
		Chironomidae	*Chironomus tepperi*	44	(Hughes et al., 2005)
		Tipullidae	*Tipula paludosa*	NA	(Oestergaard et al., 2007)
	Cry4Aa/Cyt2Ba	Culicidae	*Aedes aegypti*	NA	(Manasherob et al., 2006)
	Cry4A/Mtx1	Culicidae	*Culex quinquefasciatus*	1.06-2.37	(Wirth et al., 2014)
	Cry4A/Mtx2	Culicidae	*Culex quinquefasciatus*	0.268-1.21	(Wirth et al., 2014)
	cry4Ba/Cry11Aa	Culicidae	*Anopheles albimanus*	0.567	(Fernandez-Luna et al., 2010)
	Cry4Ba/Cyt1Aa	Culicidae	*Aedes aegypti*	0.62	(Crickmore et al., 1995a)
		Culicidae	*Anopheles albimanus*	0.333-0.767	(Fernandez-Luna et al., 2010)
	Cry4Ba/Cyt2Aa2	Culicidae	*Aedes aegypti*	0.007	(Promdonkoy et al., 2005)
			Culex quinquefasciatus	0.02	(Promdonkoy et al., 2005)
	Cry4B/Mtx1	Culicidae	*Culex quinquefasciatus*	18.2-29.0	(Wirth et al., 2014)
	Cry4B/Mtx2	Culicidae	*Culex quinquefasciatus*	85.7	(Wirth et al., 2014)
	Cry10Aa/Cyt1Aa	Culicidae	*Aedes aegypti*	0.0286-0.0818	(Hernández-Soto et al., 2009)
	Cry11Aa/Cyt1Aa	Culicidae	*Aedes aegypti*	0.0112-0.118	(Crickmore et al., 1995; Wu et al., 1994b)
		Culicidae	*Anopheles albimanus*	0.283-0.367	(Fernandez-Luna et al., 2010)
	Cry11/Mtx1	Culicidae	*Culex quinquefasciatus*	0.664-0.303	(Wirth et al., 2014)
	Cry11/Mtx2		*Culex quinquefasciatus*	0.904	(Wirth et al., 2014)
	Cry11Bb/Cry29Aa	Culicidae	*Aedes aegypti*	3.939	(Juárez-pérez et al., 2003)
			Anopheles stephensi	2.131	(Juárez-pérez et al., 2003)
			Culex pipiens	0.726	(Juárez-pérez et al., 2003)
	Cry11Bb/Cry30Aa	Culicidae	*Aedes aegypti*	16.962	(Juárez-pérez et al., 2003)
			Anopheles stephensi	1.435	(Juárez-pérez et al., 2003)
			Culex pipiens	1.13	(Juárez-pérez et al., 2003)
	Cry4A/Cry4B/Cry11A	Culicidae	*Aedes aegypti*	0.125	(Crickmore et al., 1995)
			Culex quinquefasciatus	0.0082-0.590	(Wirth et al., 2014)
	Cry4A/Cry4B/Cyt1A	Culicidae	*Aedes aegypti*	0.077	(Crickmore et al., 1995)
	Cry4A/Cry4B/Mtx1	Culicidae	*Culex quinquefasciatus*	0.181-0.769	(Wirth et al., 2014)
	Cry4A/Cry4B/Mtx2	Culicidae	*Culex quinquefasciatus*	0.114-0.324	(Wirth et al., 2014)
	Cry4A/Cry4B/Cry11A/Cyt1A	Culicidae	*Aedes aegypti*	0.085	(Crickmore et al., 1995)
			Culex quinquefasciatus	0.020-0.0753	(Wirth et al., 2014)
	Cry4A/Cry4B/Cry11A/Mtx1	Culicidae	*Culex quinquefasciatus*	0.0192-0.244	(Wirth et al., 2014)
	Cry4A/Cry4B/Cry11A/Mtx2	Culicidae	*Culex quinquefasciatus*	0.0295-0.0564	(Wirth et al., 2014)
	Cry4A/Cry4B/Cry11A/Cyt1A/Mtx1	Culicidae	*Culex quinquefasciatus*	0.0228-0.0643	(Wirth et al., 2014)
	Cry4A/Cry4B/Cry11A/Cyt1A/Mtx2	Culicidae	*Culex quinquefasciatus*	0.298-1.09	(Wirth et al., 2014)
	Cry4Ba/Cry11Aa/Cyt1Aa	Culicidae	*Anopheles albimanus*	0.7-8.33	(Fernandez-Luna et al., 2010)

Protein		Insect		Activity range LC50 (µg/ml)	References
Family	**Current Name**	**Family**	**Species**		
	Cry11Bb/Cry29Aa/Cry30Aa	Culicidae	*Aedes aegypti*	5.432	(Juárez-pérez et al., 2003)
			Anopheles stephensi	1.314	(Juárez-pérez et al., 2003)
			Culex pipiens	0.849	(Juárez-pérez et al., 2003)

4. Bt-BASED INSECTICIDES AGAINST DIPTERA

4.1 Bt-based commercial products

Organic insecticides are highly effective but carry a series of side effects, such as harmful effects on wildlife and beneficial non-target insects, contamination of water and food sources, and at the end, the appearance of insecticide-related resistances due to the selection pressure generated by repeated use with the same active ingredient or other that has a similar mode of action. The emergence of resistances is notorious, estimating that around 400 species have already developed resistance to at least one chemical insecticide (Tabashnik, 2008). Moreover, at least 17 different species have developed resistance to all classes of insecticides (Kumar et al., 2008). Due to said increasing occurrence of insect resistance to chemical insecticides, there is a real need to develop other control methods. Microbial pesticides, which constitute a series of leading products made from naturally occurring or genetically modified insecticidal bacteria, have attracted increasing attention as a specific means of controlling agricultural, forestry and sanitary pests. Bt is the most competitive microbial insecticide in terms of production costs. Although a very efficient fermentation process of Bt is required, its production is competitive against synthetic insecticides. Bt has been successfully used for decades to control agricultural pests and human disease vectors like mosquitos, because it is an environmentally friendly and highly specific biopesticide (Bravo et al., 2011; Kamareddine, 2012; Promdonkoy et al., 2005). Bt-based insecticides represent around 80% of all biopesticides in the market (Whalon and Wingerd, 2003). The specific toxicity of crystal proteins against target insects is the basis for the use of Bt as a biopesticide in agriculture, forestry and mosquito control since 1961 (Kaur, 2000). The advantages of Bt over synthetic pesticides include lack of toxic residues, high specificity to target insects, safety to non-target organisms such as mammals, birds, amphibians and reptiles, as well as low costs of development and registration (Kaur, 2000).

Genetic improvement of Bt strains for development of novel biopesticides entails increasing their potency against target insects, broadening the insecticidal spectra for specific crop applications, improving persistence on plants and optimizing fermentation production (Burges, 1998).We have to keep in mind that vegetative Bt cells have a strong ability to harbour DNA plasmids (Zhong et al., 2011) and that they are able to carry more than one *cry* gene and plasmid per strain (Kronstad et al.,

1983). Several studies reveal that the potential that exists in new Bt strains coming from different regions is huge, through the detection and characterization of large number of different genes encoding insecticidal active substances (Chattopadhyay and Banerjee, 2018). In the last few years, there have been great advances in next-generation sequencing and new "omics" technologies such as proteomics, genomics, metabolomics and transcriptomics (Chattopadhyay and Banerjee, 2018). These technologies are providing a great knowledge about the whole biology of Bt and the diversity existing between different Bt strains with different characteristics and toxicities (Dong et al., 2016).For this reason, the projects of Bt genome sequencing are increasing, and probably, they will accelerate in a remarkable way the detection of new pathogenic genes and their regulatory mechanisms (Jouzani et al., 2017).

Formulation technology must be considered at all stages from production of an organism to its eventual action on the target. The formulation of a Bt compound has four functions: 1) to stabilize the organism during production, distribution and storage; 2) to aid handling and application of the product; 3) to protect the agent from harmful environmental factors at the target site, thereby increasing persistence; 4) and to enhance the activity of the organism at the target site by increasing its activity, reproduction, contact and interaction with the target pest or disease (Burges, 1998).

There are a wide variety of formulation types, both liquid and solid. The main types currently used for organisms have been classified by Rhodes (1993) into dry products (dusts, granules and briquettes) and suspensions (oil- or water-based and emulsions). Liquid suspensions usually use water or oil, but solvents are also applied. The commonest are suspension concentrates and emulsions, although there are also specialized types such as microcapsules (Burges, 1998).

One of the most common forms of Bt application is through sprays based on spores and crystals mixtures. In fact, there are more than 98 sprayable bacterial formulations (Lacey et al., 2015). Nevertheless, these products present some drawbacks that can reduce their effectiveness as control agents in the field. One of them is the degradation suffered by the crystals due to the UV radiation of the sun and also the easy washing with rainwater (Griego and Spence, 1978; Manasherob et al., 2002). Bt products are mixed with different compounds, such as wetting agents, stickers, phagostimulants

and sunscreens, in their formulation trying to reduce the disadvantages. The fact that the production of Bt is easy and cheap (Lacey et al., 2015), together with its fast toxic action and the great host specificity, without adverse effect for non-target organism, makes Bt the most successful microbial insecticide used against both in agro-forestry pests as well as pests of veterinary-medical interest. Through the technology of the formulation and the possibility of incorporating certain adjuvants, substantial improvements in the performance of these Bt bioplaguicides have been achieved (Burges, 1998). Many new 'spreaders and stickers' have been developed to help distribute and bind Bt material evenly over the often waxy leaf surface to increase Bt persistence on plants (Behle et al., 1997). Certain feeding stimulants enhance activity of Bt against pests by causing a more rapid ingestion of the lethal dose (Farrar and Ridgway, 1995). Efficacy of Bt toxins can also be increased by using inexpensive additives such as tannic acid (Gibson et al., 1995).

4.2 Bacterial insecticides against Diptera

Currently, the major alternative for mosquito and blackfly larval control is based on bacterial toxins produced by Bti and *L. sphaericus*. Products based on Bti and *L. sphaericus* are marketed and are widely used in the US and Europe. Many different formulations have been developed. The main commercial products are suspension concentrates, followed by wettable powders and to a lesser extent by large-grained formulations. *L. sphaericus*, because of its better residual activity in polluted waters, has been broadly used against *Culex* species in the US, Central America, brazil, India, Thailand and China (Wirth, 2010). For control of mosquito larvae, formulated bacteria are sprayed or spread over the surface of static or slow-moving water into which they sink at a rate determined by the design of the formulation. Blackfly larvae live in fast-moving water courses and are controlled by pouring bacterial suspensions into the water at consecutive points, from which they are carried downstream (Burges, 1998). Different feeding habits of larvae of different species influence the effectiveness of the bacteria in mosquitoes. *Culex* larvae filter-feed up and down the column of water; they are often termed column feeders, while *Aedes* larvae tend to scavenge along substrate surfaces, particularly on the bottom. *Anopheles* larvae feed on buoyant material trapped at or just below the water surface. In comparable conditions, two *Anopheles* species filtered water at the rate of 33-34 and 49-55 µl/larva/h, respectively, while *C. quinquefasciatus* filtered 490-590 and *A. aegypti* 590-690 µl/larva/h (Aly,

1988). Larval feeding habits partly explain why species of *Anopheles* have consistently appeared less susceptible to Bt suspensions than the column- and bottom-feeding *Culex* and *Aedes* larvae in laboratory assays and field tests (Lacey and Smittle, 1985). Thus, differently formulated products are required for mosquito larvae of different feeding types. Buoyant products are required for anophelines, but products should stay in suspension below the surface for column and bottom-feeders. In natural waters, rapid sinking should be avoided because steady deposit of debris would soon cover the particles (Burges, 1998).

The effectiveness of many bacterial formulations against both mosquitoes and blackflies is short-lived in the field, often only 1-2 days. This is due to rapid settling, adsorption to plants and other substrates (which also filter particles out of the water), denaturing of the crystal by sunlight and engulfment by filter feeding fauna (Lacey, 1986; Lacey and Smittle, 1985). A major goal of the formulation process is to extend the effective period. However, UV radiation inside the water is not as important as particle settling, which is the key factor in determining the effectiveness of a given formulation, since water filters out much of the UV radiation. With Bt, only the effect of sunlight on the crystal reduces larval mortality, since the spore is unimportant in mosquito and blackfly larvae. *L. sphaericus* is more susceptible to sunlight, being inactivated in clear water a few centimetres deep in full sun (Mulligan et al., 1980), while strong sunlight reduces its effectiveness several-fold (Skovmand and Bauduin, 1998). A sunscreen might be beneficial with *L. sphaericus*, particularly in formulations designed to float.

To increase the effectiveness of active Cry proteins against Diptera, they have been transferred to alternative administration hosts to increase their persistence in aquatic feeding areas. An improved biopesticide for mosquitoes was developed by inserting *cry* genes from Bti, which is highly toxic to mosquitoes, into the chromosome of *L. sphaericus*, which has longer environmental persistence (Bar et al., 1998). The chromosomally integrated *cry* genes were maintained through several generations in the absence of selective pressure. The recombinant *L. sphaericus* producing high levels of *cry11A* gene product from Bti was toxic to *Aedes*, *Culex* and *Anopheles* larvae (Poncet et al., 1997).

4.3 Species of Diptera resistant to Bt

Despite the success of Bt-based insecticides for the control of mosquito larvae and their safety for the environment, these biopesticides may become ineffective if resistances to the insecticide appear. Due to the strong pressure of directional selection exerted by the continued and repeated use of an insecticide, resistance phenomena to these formulations can occur. The individual insects of a population subjected to treatments may naturally possess genetic characteristics that reduce their susceptibility to an insecticide. These individuals survive treatment in greater proportion than individuals who are more susceptible to treatment, and consequently in later generations, their offspring will be present in a greater proportion. Over time, with repeated treatments, the frequency of resistant individuals increases and the population may become difficult to control until the insecticide, and closely related insecticides, are useless for significantly reducing population numbers (Wirth, 2010). The rapid development of resistance is one of the main problems in the control of insects with chemical insecticides. In contrast to this, the possibility of the rapid development of resistance against microbial control agents seems to be unlikely to the same extent as the complex mode of action between pathogens and target organism increases (Davidson, 1992). Nevertheless, resistance against microbial insecticides is possible in principle. Tabashnik and collaborators have shown that routine treatments with Bt ser. *kurstaki* products in agriculture can lead to a significant resistance within a few years (Ferré et al., 1991; Tabashnik et al., 1990).

Several attempts to analyse resistances to Bti in different species and populations of mosquitoes under laboratory conditions have been undertaken, and the results do not seem to provide evidence of resistance to these compounds. Some studies showed that there was a slight decrease in the susceptibility to Bti over several generations. However, when the selection pressure was stopped, the generated resistance disappeared (Gharib and Szalay-Marzso, 1986; Goldman et al., 1986; Vasquez et al., 2009). These works have been carried out with *A. aegypti* and *C. quinquefasciatus* as model species, using LC_{50} or LC_{95} values for the selection pressure (Georghiou, 1983). Regardless of the origin of the collection or the level of selection pressure, only modest levels of resistance were recorded. No data are available about the resistance phenomena of mosquito field populations treated with Bti. Despite this, some authors showed that there was not increase in resistance to black flies

(Simulium damnosum) after 7 years of exclusive control using Bti (Kurtak et al., 1987). There may be several reasons why resistance was not found in mosquitoes, in spite of years of extensive control of floodwater mosquitoes with Bti. 1) Short exposure period of the toxins. The period from when the insecticide is applied, until the death of the target insect is achieved is short. 2) Bti mode of action. It is assumed that the lethal changes within the cells of the midgut are produced by the synergistic effects of different proteins of the parasporal body (Federici et al., 1990). 3) Intrinsic characteristics of the target mosquito population. The variability in the genes of the target insect population due to the migration within the breeding areas, producing a constant gene flow, which at least delays the development of resistance (Becker, 1989).

Therefore, Bti in its native form shows a unique capacity to avoid the evolution of resistance in mosquito populations compared to most conventional insecticides. Although most studies have been mainly carried out in *C. quinquefasciatus*, there are also studies with *A. aegypti* and *C. pipiens* that corroborate this, which suggests that it may be true for other mosquito species. The studies using recombinant Bti strains provide the strongest evidence that it is the complex mixture of toxins and their specific interactions that reduce the risk for resistance, most importantly the presence of Cyt1A (Georghiou and Gibbons, 1986; Wirth and Georghiou, 1997). However, a study with a Bti-selected line of *C. quinquefasciatus* indicates that there is a cross-resistance observed against Cry protein. This resistance is suppressed by interactions with other Cry proteins, and is strongly suppressed by the presence of Cyt1A protein (Wirth, 2010). Accordingly, given the number of different mosquito populations and species that may be targeted with Bti, it cannot be concluded that resistance will not occur, but that the relative risk is low (Crickmore et al., 1995; Georghiou and Wirth, 1997; S. Poncet et al., 1995; Wirth and Georghiou, 1997).

5. AIMS OF THE THESIS

The main aims of this thesis were:

1. Adaptation of a quantitative method for determining the toxic properties of insecticidal compounds or pathogens that act by ingestion in adult Diptera that feed by consuming droplets.
2. To determine the functional importance of the proteins which are minor components of the crystal produced by certain Bti strains.

In order to accomplish these two aims of the thesis, the following specific activities will be addressed:

1. Estimate the volume ingested by flies of each sex and perform a proof-of-concept study using the naturally-derived insecticide spinosad to determine the concentration-mortality, dose-mortality and dose per mg body weight relationship.
2. Determine the toxic activity of Bti minor proteins Cry10A and Cyt2B against *A. aegypti* and *C. capitata*.
3. Analysis of the possible synergistic interaction between the two minor proteins in the toxicity against *A. aegypti*.

6. REFERENCES

Abdul-Rauf, M., Ellar, D.J., 1999. Toxicity and receptor binding properties of a *Bacillus thuringiensis* CryIC Toxin active against Both Lepidoptera and Diptera. J. Invertebr. Pathol. 73, 52–58. https://doi.org/10.1006/jipa.1998.4793

Abdullah, M.A.F., Alzate, O., Mohammad, M., McNall, R.J., Adang, M.J., Dean, D.H., 2003. Introduction of Culex toxicity into *Bacillus thuringiensis* Cry4Ba by protein engineering. Appl. Environ. Microbiol. 69, 5343–5353. https://doi.org/10.1128/AEM.69.9.5343-5353.2003

Adams, L.F., Visick, J.E., Whiteley, H.R., 1989. A 20-kilodalton protein is required for efficient production of the *Bacillus thuringiensis* subsp. *israelensis* 27-kilodalton crystal protein in *Escherichia coli*. J. Bacteriol. 171, 521–530. https://doi.org/10.1128/jb.171.1.521-530.1989

Ahmad, W., Nicholls, C., Ellar, D.J., 1989. Cloning and expression of an entomocidal protein gene from *Bacillus thuringiensis galleriae* toxic to both lepidoptera and diptera. FEMS Microbiol. Lett. 59, 197–201. https://doi.org/10.1111/j.1574-6968.1989.tb03109.x

Al-yahyaee, S.A.S., Ellar, D.J., 1995. Maximal toxicity of cloned CytA δ-endotoxin from *Bacillus thuringiensis* subsp. *israelensis* requires proteolytic processing from both the N- and C-termini. Microbiology 141, 3141–3148. https://doi.org/10.1099/13500872-141-12-3141

Allende, A., 2016. Risks for public health related to the presence of *Bacillus cereus* and other Bacillus spp. including *Bacillus thuringiensis* in foodstuffs. EFSA J. 14. https://doi.org/10.2903/j.efsa.2016.4524

Aly, C., 1988. Filtration rates of mosquito larvae in suspensions of latex microspheres and yeast cells. Entomol. Exp. Appl. 46, 55–61. https://doi.org/10.1111/j.1570-7458.1988.tb02267.x

Anderson, I., Sorokin, A., Kapatral, V., Reznik, G., Bhattacharya, A., Mikhailova, N., Burd, H., Joukov, V., Kaznadzey, D., Walunas, T., D'Souza, M., Larsen, N., Pusch, G., Liolios, K., Grechkin, Y., Lapidus, A., Goltsman, E., Chu, L., Fonstein, M., Ehrlich, S.D., Overbeek, R., Kyrpides, N., Ivanova, N., 2005. Comparative genome analysis of *Bacillus cereus* group genomes with *Bacillus subtilis*. FEMS Microbiol. Lett. 250, 175–184. https://doi.org/10.1016/j.femsle.2005.07.008

Angsuthanasombat, C., Crickmore, N., Ellar, D.J., 1992. Comparison of *Bacillus thuringiensis* subsp. *israelensis* CryIVA and CryIVB cloned toxins reveals synergism in vivo. FEMS Microbiol. Lett. 94, 63–68. https://doi.org/10.1016/0378-1097(92)90584-B

Angsuthanasombat, C., Panyim, S., 1989. Biosynthesis of 130-kilodalton mosquito larvicide in the cyanobacterium *Agmenellum quadruplicatum*. PR-6. Appl. Environ. Microbiol. 55, 2428–2430.

Angusthanasombat, C., Crickmore, N., Ellar, D.J., 1991. Cytotoxicity of a cloned *Bacillus thuringiensis* subsp. *israelensis* CryIVB toxin to an *Aedes aegypti* cell line. FEMS Microbiol. Lett. 83, 273–276. https://doi.org/10.1111/j.1574-6968.1991.tb04476.x

Bar, E., Sandler, N., Makayoto, M., Keynan, A., 1998. Expression of Chromosomally Inserted *Bacillus thuringiensis israelensis* Toxin Genes in *Bacillus sphaericus*. J. Invertebr. Pathol. 72, 206–213. https://doi.org/10.1006/jipa.1998.4787

Barloy, F., Delécluse, A., Nicolas, L., Lecadet, M.M., 1996. Cloning and expression of the first anaerobic toxin gene from *Clostridium bifermentans* subsp. *malaysia*, encoding a new mosquitocidal protein with homologies to *Bacillus thuringiensis* δ-endotoxins. J. Bacteriol. 178, 3099–3105. https://doi.org/10.1128/jb.178.11.3099-3105.1996

Baumann, L., Broadwell, A.H., Baumann, P., 1988. Sequence analysis of the mosquitocidal toxin genes encoding 51.4- and 41.9-kilodalton proteins from *Bacillus sphaericus* 2362 and 2297. J. Bacteriol. 170, 2045–2050. https://doi.org/10.1128/jb.170.5.2045-2050.1988

Becker, N., 1989. Life strategies of mosquitoes as an adaptation to their habitats. Bull. Soc. Vector Ecol. 14, 6–25.

Behle, R.W., McGuire, M.R., Shasha, B.S., 1997. Effects of Sunlight and Simulated Rain on Residual Activity of

Bacillus thuringiensis Formulations. J. Econ. Entomol. 90, 1560–1566. https://doi.org/10.1093/jee/90.6.1560

Beltrão, H., Silva-Filha, M.H.N.L., 2007. Interaction of *Bacillus thuringiensis* var. *israelensis* Cry toxins with binding sites from *Aedes aegypti* (Diptera: Culicidae) larvae midgut. FEMS Microbiol. Lett. 266, 163–169. https://doi.org/10.1111/j.1574-6968.2006.00527.x

Ben-Dov, E., 2014. *Bacillus thuringiensis* subsp. *israelensis* and Its dipteran-specific toxins. Toxins (Basel). 6, 1222–1243. https://doi.org/10.3390/toxins6041222

Berliner, E., 1915. Ueber die schlaffsucht der *Ephestia kuhniella* und *Bac. thuringiensis* n. sp. Z Angew Entomol 2, 21–56.

Bernhard, K., Jarrett, P., Meadows, M., Butt, J., Ellis, D.J., Roberts, G.M., Pauli, S., Rodgers, P., Burges, H.D., 1997. Natural Isolates of *Bacillus thuringiensis*: Worldwide Distribution, Characterization, and Activity against Insect Pests 68, 59–68.

Berón, C.M., Salerno, G.L., 2007. Cloning and characterization of a novel crystal protein from a native *Bacillus thuringiensis* isolate highly active against *Aedes aegypti*. Curr. Microbiol. 54, 271–276. https://doi.org/10.1007/s00284-006-0299-8

Berry, C., 2012. The bacterium , *Lysinibacillus sphaericus* , as an insect pathogen. J. Invertebr. Pathol. 109, 1–10. https://doi.org/10.1016/j.jip.2011.11.008

Berry, C., Ben-dov, E., Jones, A.F., Murphy, L., Quail, M.A., Holden, M.T.G., Harris, D., Zaritsky, A., Parkhill, J., 2002. Complete Sequence and Organization of pBtoxis, the Toxin-Coding Plasmid of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 68, 5082–5095. https://doi.org/10.1128/AEM.68.10.5082

Bietlot, H.P.L., Vishnubhatla, I., Carey, P.R., Pozsgay, M., Kaplan, H., 1990. Characterization of the cysteine residues and disulphide linkages in the protein crystal of *Bacillus thuringiensis*. Biochem. J. 267, 309–315. https://doi.org/10.1042/bj2670309

Blackburn, M.B., Martin, P.A.W., Kuhar, D., Farrar, R.R., Gundersen-Rindal, D.E., 2013. Phylogenetic Distribution of Phenotypic Traits in *Bacillus thuringiensis* Determined by Multilocus Sequence Analysis. PLoS One 8. https://doi.org/10.1371/journal.pone.0066061

Bourgouin, C., Delecluse, A., Ribier, J., Klier, A., Rapoport, G., 1988. A *Bacillus thuringiensis* subsp. *israelensis* Gene Encoding a 125-Kilodalton Larvicidal Polypeptide Is Associated with Inverted Repeat Sequences. J. Bacteriol. 170, 3575–3583.

Bravo, A., Gómez, I., Conde, J., Muñoz-Garay, C., Sánchez, J., Miranda, R., Zhuang, M., Gill, S.S., Soberón, M., 2004. Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. Biochim. Biophys. Acta - Biomembr. 1667, 38–46. https://doi.org/10.1016/j.bbamem.2004.08.013

Bravo, A., Gómez, I., Porta, H., García-Gómez, B.I., Rodriguez-Almazan, C., Pardo, L., Soberón, M., 2013. Evolution of *Bacillus thuringiensis* Cry toxins insecticidal activity. Microb. Biotechnol. 6, 17–26. https://doi.org/10.1111/j.1751-7915.2012.00342.x

Bravo, A., Likitvivatanavong, S., Gill, S.S., Soberon, M., 2011.*Bacillus thuringiensis*: A story of a successful bioinsecticide. Insect Biochem. Mol. Biol. 41, 423–431.

Bravo, A., Sarjeet, G., Mario, S., 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and Their Potential for Insect Control. Natl. Institutes Acess 49, 423–435. https://doi.org/10.1016/j.toxicon.2006.11.022

Bukhari, D.A.A., Shakoori, A.R., 2009. Cloning and expression of *Bacillus thuringiensis* Cry11 crystal protein gene in *Escherichia coli*. Mol. Biol. Rep. 36, 1661–1670. https://doi.org/10.1007/s11033-008-9366-5

Burges, H.D., 1998. Formulation of Microbial Biopesticides. Beneficial microorganisms, nematodes and seed treatments.Kluwer academic publishers.

Butko, P., 2003. Cytolytic toxin Cyt1A and its mechanism of membrane damage: data and hypotheses. Appl. Environ. Microbiol. 69, 2415–2422. https://doi.org/10.1128/AEM.69.5.2415-2422.2003

Butko, P., Huang, F., Pusztai-Carey, M., Surewicz, W.K., 1997. Interaction of the δ-endotoxin CytA from *Bacillus thuringiensis* var. *israelensis* with lipid membranes. Biochemistry 36, 12862–12868. https://doi.org/10.1021/bi9702389

Butko, P., Huang, F., Pusztai-Carey, M., Surewicz, W.K., 1996. Membrane permeabilization induced by cytolytic δ-endotoxin CytA from *Bacillus thuringiensis* var. *israelensis*. Biochemistry 35, 11355–11360. https://doi.org/10.1021/bi960970s

Caballero, P., Ferré, J., 2001. Bioinsecticidas: fundamentos y aplicaciones de *Bacillus thuringiensis* en el control integrado de plagas. Phytoma. España.

Cantón, P.E., Reyes, E.Z., De Escudero, I.R., Bravo, A., Soberón, M., 2011. Binding of *Bacillus thuringiensis* subsp. *israelensis* Cry4Ba to Cyt1Aa has an important role in synergism. Peptides 32, 595–600. https://doi.org/10.1016/j.peptides.2010.06.005

Carpusca, I., Jank, T., Aktories, K., 2006. *Bacillus sphaericus* mosquitocidal toxin (MTX) and pierisin : the enigmatic offspring from the family of ADP-ribosyltransferases 62, 621–630. https://doi.org/10.1111/j.1365-2958.2006.05401.x

Chan, S.W., Thanabalu, T., Wee, B.Y., Porter, A.G., 1996. Unusual amino acid determinants of Host Range in the Mtx2 Family of Mosquitocidal Toxins. J. Biol. Chem. 271, 14183–14187.

Chang, C., Dai, S.M., Frutos, R., Federici, B.A., Gill, S.S., 1992. Properties of a 72-kilodalton mosquitocidal protein from *Bacillus thuringiensis* subsp. *morrisoni* PG-14 expressed in *B. thuringiensis* subsp. *kurstaki* by using the shuttle vector pHT3101. Appl. Environ. Microbiol. 58, 507–512.

Chang, C., Yu, Y.M., Dai, S.M., Law, S.K., Gill, S.S., 1993. High-level cryIVD and cytA gene expression in *Bacillus thuringiensis* does not require the 20-kilodalton protein, and the coexpressed gene products are synergistic in their toxicity to mosquitoes. Appl. Environ. Microbiol. 59, 815–821.

Chattopadhyay, P., Banerjee, G., 2018. Recent advancement on chemical arsenal of Bt toxin and its application in pest management system in agricultural field. 3 Biotech 8, 1–12. https://doi.org/10.1007/s13205-018-1223-1

Chaufaux, J., Muller-Cohn, J., Buisson, C., Sanchis, V., Lereclus, D., Pasteur, N., 1997. Inheritance of Resistance to the *Bacillus thuringiensis* CrylC Toxin in *Spodoptera littoralis* (Lepidoptera: Noctuidae). J. Econ. Entomol. 90, 873–878. https://doi.org/10.1093/jee/90.4.873

Chen, J., Aimanova, K.G., Fernandez, L.E., Bravo, A., Soberon, M., Gill, S.S., 2009a. *Aedes aegypti* cadherin serves as a putative receptor of the Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis*. Biochem. J. 424, 191–200. https://doi.org/10.1042/BJ20090730.

Chen, J., Aimanova, K.G., Pan, S., Gill, S.S., 2009b. Identification and characterization of *Aedes aegypti* aminopeptidase N as a putative receptor of *Bacillus thuringiensis* Cry11A toxin. Insect Biochem. Mol. Biol. 39, 688–696. https://doi.org/10.1016/j.ibmb.2009.08.003

Cheong, H., Dhesi, R.K., Gill, S.S., 1997. Marginal cross-resistance to mosquitocidal *Bacillus thuringiensis* strains in Cry11A-resistant larvae: Presence of Cry11A-like toxins in these strains. FEMS Microbiol. Lett. 153, 419–424. https://doi.org/10.1016/S0378-1097(97)00283-8

Cheong, H., Gill, S.S., 1997. Cloning and characterization of a cytolytic and mosquitocidal δ- endotoxin from *Bacillus thuringiensis* subsp. *jegathesan*. Appl. Environ. Microbiol. 63, 3254–3260.

Chilcott, C., Ellar, D., 1988. Comparative Toxicity of *Bacillus thuringiensis* var. *israelensis* crystal proteins in vivo and in vitro. J. Gen. Microbiol. 134, 2551–2558.

Chilcott, C.N., Wigley, P.J., 1994. Opportunities for finding new *Bacillus thuringiensis* strains. Agric. Ecosyst. Environ. 49, 51–57. https://doi.org/10.1016/0167-8809(94)90022-1

Chilcott, C.N., Wigley, P.J., Broadwell, A.H., Park, D.J., Ellar, D.J., 1998. Activities of *Bacillus thuringiensis* insecticidal crystal proteins Cyt1Aa and Cyt2Aa against three species of sheep blowfly. Appl. Environ. Microbiol. 64, 4060–4061.

Cohen, S., Albeck, S., Ben-Dov, E., Cahan, R., Firer, M., Zaritsky, A., Dym, O., 2011. Cyt1Aa toxin: Crystal structure reveals implications for its membrane-perforating function. J. Mol. Biol. 413, 804–814. https://doi.org/10.1016/j.jmb.2011.09.021

Cohen, S., Dym, O., Albeck, S., Ben-Dov, E., Cahan, R., Firer, M., Zaritsky, A., 2008. High-Resolution Crystal Structure of Activated Cyt2Ba Monomer from *Bacillus thuringiensis* subsp. *israelensis*. J. Mol. Biol. 380, 820–827. https://doi.org/10.1016/j.jmb.2008.05.010

Couche, G.A., Pfannenstiel, M.A., Nickerson, K.W., 1987. Structural disulfide bonds in the *Bacillus thuringiensis* subsp. *israelensis* protein crystal. J. Bacteriol. 169, 3281–3288. https://doi.org/10.1128/jb.169.7.3281-3288.1987

Crickmore, N., 2013. The diversity of *Bacillus thuringiensis* d-endotoxins. Entomopathog. Bact. from Lab. to F. Appl. 65–79.

Crickmore, N., Bone, E.J., Williams, J.A., Ellar, D.J., 1995. Contribution of the individual components of the δ-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. FEMS Microbiol. Lett. 131, 249–254. https://doi.org/10.1016/0378-1097(95)00264-6

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Dean, D.H., 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62, 807–13.

Daffonchio, D., Brusetti, L., Brusetti, L., Rizzi, A., Rizzi, A., Chanishvili, N., Chanishvili, N., Visca, P., Visca, P., Sharp, R., Sharp, R., Borin, S., Borin, S., 2006. Strategy for Identification of *Bacillus cereus* and *Bacillus thuringiensis* Strains Closely Related to *Bacillus anthracis*. Society 72, 1295–1301. https://doi.org/10.1128/AEM.72.2.1295

Dai, S. mei, Gill, S.S., 1993. In vitro and in vivo proteolysis of the *Bacillus thuringiensis* subsp. *israelensis* CryIVD protein by *Culex quinquefasciatus* larval midgut proteases. Insect Biochem. Mol. Biol. 23, 273–283. https://doi.org/10.1016/0965-1748(93)90008-G

Davidson, E.W., 1992. Development of insects resistence to biopesticides. Pesqui. Agropecuária Bras. 27, 47-57.

de Barjac, H., Bonnefoi, A., 1962. Essai de Classificaton biochimique et serologique de 24 souches de bacillus du type *B. thuringiensis*. Entomophaga 7, 5–31.

de Barjac, H., Frachon, E., 1990. Classification of *Bacillus thuringiensis* strains. Entomophaga 35, 233–240. https://doi.org/10.1007/BF02374798

de Maagd, R.A., Bosch, D., Stiekema, W., 1999. *Bacillus thuringiensis* toxin-mediated insect resistance in plants. Trends Plant Sci. 4, 9–13. https://doi.org/10.1016/S1360-1385(98)01356-9

de Maagd, R.A., Bravo, A., Berry, C., Crickmore, N., Schnepf, H.E., 2003. Structure, Diversity, and Evolution of Protein Toxins from Spore-Forming Entomopathogenic Bacteria. Annu. Rev. Genet. 37, 409–433. https://doi.org/10.1146/annurev.genet.37.110801.143042

de Maagd, R.A., Bravo, A., Crickmore, N., 2001. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends Genet. 17, 193–199. https://doi.org/10.1016/S0168-9525(01)02237-5

Delécluse, A., Bourgouin, C., Klier, A., Rapoport, G., 1988. Specificity of action on mosquito larvae of *Bacillus thuringiensis israelensis* toxins encoded by two different genes. MGG Mol. Gen. Genet. 214, 42–47. https://doi.org/10.1007/BF00340177

Delecluse, A., Poncet, S., Klier, A., Rapoport, G., 1993. Expression of cryIVA and cryIVB Genes, Independently or in Combination, in a Crystal-Negative Strain of *Bacillus thuningiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 59, 3922–3927.

Delecluse, A., Rosso, M.L., Ragni, A., 1995. Cloning and expression of a novel toxin gene from *Bacillus thuringiensis* subsp. *jegathesan* encoding a highly mosquitocidal protein. Appl. Environ. Microbiol. 61, 4230–4235.

Dervyn, E., Poncet, S., Klier, A., Rapoport, G., 1995. Transcriptional regulation of the cryIVD gene operon from

Bacillus thuringiensis subsp. *israelensis*. J. Bacteriol. 177, 2283–2291. https://doi.org/10.1128/jb.177.9.2283-2291.1995

Dong, Z., Li, J., Zheng, J., Geng, C., Peng, D., Sun, M., 2016. Complete genome sequence of *Bacillus thuringiensis* CTC-A typical strain with high production of S-layer proteins. J. Biotechnol. 220, 100–101. https://doi.org/10.1016/j.jbiotec.2015.12.027

Donovan, W.P., Dankocsik, C., Gilbert, M.P., 1988. Molecular characterization of a gene encoding a 72-kilodalton mosquito-toxic crystal protein from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. 170, 4732–4738. https://doi.org/10.1128/jb.170.10.4732-4738.1988

Donovan, W.P., Donovan, J.C., Engleman, J.T., 2001. Gene knockout demonstrates that vip3A contributes to the pathogenesis of *Bacillus thuringiensis* toward *Agrotis ipsilon* and *Spodoptera exigua*. J. Invertebr. Pathol. 78, 45–51. https://doi.org/10.1006/jipa.2001.5037

Donovan, W.P., Engleman, J.T., Donovan, J.C., Baum, J.A., Bunkers, G.J., Chi, D.J., Clinton, W.P., English, L., Heck, G.R., Ilagan, O.M., Krasomil-Osterfeld, K.C., Pitkin, J.W., Roberts, J.K., Walters, M.R., 2006. Discovery and characterization of Sip1A: A novel secreted protein from *Bacillus thuringiensis* with activity against coleopteran larvae. Appl. Microbiol. Biotechnol. 72, 713–719. https://doi.org/10.1007/s00253-006-0332-7

Dulmage, H.T., 1981. Insecticidal activity of isolated of *Bacillus thuringiensis* and their potential for pest control. Microb. Control pests plant Dis. 1970-1980.

Elleuch, J., Tounsi, S., Belguith Ben Hassen, N., Lacoix, M.N., Chandre, F., Jaoua, S., Zribi Zghal, R., 2015. Characterisation of novel *Bacillus thuringiensis* isolates against *Aedes aegypti* (Diptera: Culicidae) and *Ceratitis capitata* (Diptera: Tephridae). J. Invertebr. Pathol. 124, 90–97. https://doi.org/10.1016/j.jip.2014.11.005

Estruch, J.J., Warren, G.W., Mullins, M. a, Nye, G.J., Craig, J. a, Koziel, M.G., 1996. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proc. Natl. Acad. Sci. U. S. A. 93, 5389–5394. https://doi.org/10.1073/pnas.93.11.5389

Fang, S., Wang, L., Guo, W., Zhang, X., Peng, D., Luo, C., Yu, Z., Sun, M., 2009. *Bacillus thuringiensis* bel protein enhances the toxicity of Cry1Ac protein to *Helicoverpa armigera* larvae by degrading insect intestinal mucin. Appl. Environ. Microbiol. 75, 5237–5243. https://doi.org/10.1128/AEM.00532-09

Farrar, R.R., Ridgway, R.L., 1995. Enhancement of activity of *Bacillus thuringiensis* Berliner against four Lepidopterous insect pests by nutrient-based phagostimulants. J. Entomol. Sci. 30, 29–42.

Federici, B.A., 1999. *Bacillus thuringiensis*, Handbook o. ed. Academic Press, San Diego.

Federici, B.A., Luthy, P., Ibarra, J., 1990. Parasporal body of *Bacillus thuringiensis israelensis*, in: de barjac, H., Sutherland, D. (Eds.), Bacterial Control of Mosquitoes and Black Flies. New Brunswick, NJ: Rutgers University Press, pp. 17–44.

Federici, B.A., Park, H.W., Sakano, Y., 2006. Insecticidal Protein Crystals of *Bacillus thuringiensis*., in: Shively J.M. (Eds) Inclusions in Prokaryotes. Microbiology Monographs. Microbiology Monographs, Berlin, Heidelberg.

Feldmann, F., Dullemans, A., Waalwijk, C., 1995. Binding of the CryIVD toxin of *Bacillus thuringiensis* subsp. *israelensis* to larval dipteran midgut proteins. Appl. Environ. Microbiol. 61, 2601–2605.

Fernandez-Luna, M.T., Lanz-mendoza, H., Gill, S.S., Soberon, M., Miranda-rios, J., 2010. An α-amylase is a novel receptor for *Bacillus thuringiensis* ssp. *israelensis* Cry4Ba and Cry11Aa toxins in the malaria vector mosquito *Anopheles albimanus* (Diptera: Culicidae). Environ. Microbiol. 12, 746–757. https://doi.org/10.1111/j.1462-2920.2009.02117.x.An

Fernandez, L.E., Aimanova, K.G., Gill, S.S., Bravo, A., Soberón, M., 2006. A GPI-anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae. Biochem. J. 394, 77–84. https://doi.org/10.1042/BJ20051517

Ferré, J., Real, M. D., Van, J., Jansens, S., Peferoen, M., 1991. Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. Proc. Natl. Acad. Sci. 88, 5119-5123.

Fillinger, U., Knols, B.G.J., Becker, N., 2003. Efficacy and efficiency of new *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* formulations against Afrotropical anophelines in Western Kenya. Trop. Med. Int. Heal. 8, 37–47. https://doi.org/10.1046/j.1365-3156.2003.00979.x

Florez, A.M., Suarez-Barrera, M.O., Morales, G.M., Rivera, K.V., Orduz, S., Ochoa, R., Guerra, D., Muskus, C., 2018. Toxic activity, molecular modeling and docking simulations of *Bacillus thuringiensis* Cry11 toxin variants obtained via DNA shuffling. Front. Microbiol. 9, 1–14. https://doi.org/10.3389/fmicb.2018.02461

Garduno, F., Thorne, L., Walfield, A.M., Pollock, T.J., 1988. Structural relatedness between mosquitocidal endotoxins of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 54, 277–279.

Gatehouse, J.A., 2008. Biotechnological prospects for engineering insect-resistant plants. Plant Physiol. 146, 881–887. https://doi.org/10.1104/pp.107.111096

Georghiou, G.P., 1983. Management of Resistance in arthropods. Pest Resist. to Pestic. University of California.

Georghiou, G.P., Wirth, M.C., 1997. Influence of Exposure to Single versus Multiple Toxins of *Bacillus thuringiensis* subsp. *israelensis* on Development of Resistance in the Mosquito *Culex quinquefasciatus* (Diptera: Culicidae). Appl. Environ. Microbiol. 63, 1095–1101.

Georghiou, L., Gibbons, M., 1986. Cabri Project—a Collaborative Research Facility. R&D Manag. 16, 225–232. https://doi.org/10.1111/j.1467-9310.1986.tb01306.x

Gharib, A.H., Szalay-Marzso, L., 1986. Selection for resistance to *Bacillus thuringiensis* serotype H- 14 in a laboratory strain of *Aedes aegypti*, in: R. A. Samson, J. M. Vlak and D. Peters (Eds.), Fundamental and Applied Aspects of Invertebrate Pathology, Found. Fourth Intl. Colloq. Invertebr. Pathol. Wageningen, Netherlands, p. 37.

Gibson, D.M., Gallo, L.G., Krasnoff, S.B., Ketchum, R.E., 1995. Increased efficacy of *Bacillus thuringiensis* subsp. *kurstaki* in combination with tannic acid. J. Econ. Entomol. 88, 270–277. https://doi.org/10.1093/jee/88.2.270

Gill, S.S., Singh, G.J.P., Hornung, J.M., 1987. Cell membrane interaction of *Bacillus thuringiensis* subsp. *israelensis* cytolytic toxins. Infect. Immun. 55, 1300–1308.

Gohar, M., Perchat, S., 2001. Sample preparation for β-exotoxin determination in *Bacillus thuringiensis* cultures by reversed-phase high-performance liquid chromatography. Anal. Biochem. 298, 112–117. https://doi.org/10.1006/abio.2001.5373

Goldberg, L.J., Margalit, J., 1977. A Bacterial Spore Demonstrating Rapid Larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univitattus*, *Aedes aegypti* and *Culex pipiens*. 1, 355–362.

Goldman, I.F., Arnold, J., Carlton, B.C., 1986. Selection for resistance to *Bacillus thuringiensis* subspecies *israelensis* in field and laboratory populations of the mosquito *Aedes aegypti*. J. Invertebr. Pathol. 47, 317–324. https://doi.org/10.1016/0022-2011(86)90102-3

Gonzalez, J.J., Dulmage, H.T., Carlton, B., 1981. Correlation between specific plasmids and delta-endotoxin production in *Bacillus thuringiensis*. Plasmid 5, 352–65.

Gough, J.M., Kemp, D.H., Akhurst, R.J., Pearson, R.D., Kongsuwan, K., 2005. Identification and characterization of proteins from *Bacillus thuringiensis* with high toxic activity against the sheep blowfly, *Lucilia cuprina*. J. Invertebr. Pathol. 90, 39–46. https://doi.org/10.1016/j.jip.2005.05.012

Griego, V.M., Spence, K.D., 1978. Inactivation of *Bacillus thuringiensis* spores by ultraviolet and visible light. Appl. Environ. Microbiol. 35, 906–910.

Griffitts, J.S., Haslam, S.M., Yang, T., Garczynski, S.F., Mulloy, B., Morris, H., Cremer, P.S., Dell, A., Adang, M.J., Aroian, R. V., 2005. Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin. Science. 307, 922–925. https://doi.org/10.1126/science.1104444

Guerchicoff, A., Delécluse, A., Rubinstein, C.P., 2001. The *Bacillus thuringiensis* cyt Genes for Hemolytic Endotoxins Constitute a Gene Family. Appl. Environ. Microbiol. 67, 1090–1096. https://doi.org/10.1128/AEM.67.3.1090-1096.2001

Guerchicoff, A., Ugalde, R.A., Rubinstein, C.P., 1997. Identification and characterization of a previously undescribed cyt gene in *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 63, 2716–2721. https://doi.org/10.1016/j.sse.2014.01.001

Gutierrez, P., Alzate, O., Orduz, S., 2001. A Theoretical Model of the Tridimensional Structure of *Bacillus thuringiensis* subsp. *medellin* Cry 11Bb Toxin Deduced by Homology Modelling. Mem. Inst. Oswaldo Cruz 96, 357–364.

Haider, M., Ward, E.S., Ellar, D.J., 1987. Cloning and heterologous expression of an insecticidal delta-endotoxin gene from *Bacillus thuringiensis* var. *aizawai* ICI toxic to both lepidoptera and diptera. Gene 52, 285–290.

Haider, M.Z., Ellar, D.J., 1989. Mechanism of action of *Bacillus thuringiensis* insecticidal δ-endotoxin: interaction with phospholipid vesicles. BBA - Biomembr. 978, 216–222. https://doi.org/10.1016/0005-2736(89)90118-1

Han, C., Bruce, D., Campbell, C.S., Campbell, M.L., Chen, J., Chertkov, O., Goodwin, L.A., Hill, K.K., Hitchcock, P., Jackson, P.J., Keim, P., 2006. Pathogenomic Sequence Analysis of *Bacillus cereus* and *Bacillus thuringiensis* Isolates Closely Related to *Bacillus anthracis*. J. Bac. 188, 3382–3390. https://doi.org/10.1128/JB.188.9.3382

Hayakawa, T., Yoneda, N., Okada, K., Higaki, A., Howlader, M.T.H., Ide, T., 2017. *Bacillus thuringiensis* Cry11Ba works synergistically with Cry4Aa but not with Cry11Aa for toxicity against mosquito *Culex pipiens* (Diptera: Culicidae) larvae. Appl. Entomol. Zool. https://doi.org/10.1007/s13355-016-0454-z

Heath, A.C.G., Wigley, P.J., Shoemaker, C.B., Chilcott, C.N., Broadwell, A.H., 2004. Efficacy of Native and Recombinant Cry1B Protein Against Experimentally Induced and Naturally Acquired Ovine Myiasis (Fly Strike) in Sheep. J. Econ. Entomol. 97, 1797–1804. https://doi.org/10.1603/0022-0493-97.6.1797

Helgason, E., Økstad, O.L.E.A., Caugant, D.A., Mock, L.E., Hegna, I.D. a, Johansen, H.A., Fouet, A., 2000. One Species on the Basis of Genetic Evidence. Appl. Environ. Microbiol. 66, 2627–2630. https://doi.org/10.1128/AEM.66.6.2627-2630.2000.

Hernández-Soto, A., Del Rincón-Castro, M.C., Espinoza, A.M., Ibarra, J.E., 2009. Parasporal body formation via overexpression of the Cry10Aa toxin of *Bacillus thuringiensis* subsp. *israelensis*, and Cry10Aa-Cyt1Aa synergism. Appl. Environ. Microbiol. 75, 4661–4667. https://doi.org/10.1128/AEM.00409-09

Hire, R.S., Makde, R.D., Dongre, T.K., D'souza, S.F., 2009. Expression, purification and characterization of the Cry2Aa14 toxin from *Bacillus thuringiensis* subsp. *kenyae*. Toxicon 54, 519–524. https://doi.org/10.1016/j.toxicon.2009.05.022

Höfte, H., Whiteley, H.R., 1989. Insecticidal Crystal Proteins of *Bacilllus thuringiensis*. Microbiol. Rev. 53, 242–255.

Hughes, P.A., Stevens, M.M., Park, H.W., Federici, B.A., Dennis, E.S., Akhurst, R., 2005. Response of larval *Chironomus tepperi* (Diptera: Chironomidae) to individual *Bacillus thuringiensis* var. *israelensis* toxins and toxin mixtures. J. Invertebr. Pathol. 88, 34–39. https://doi.org/10.1016/j.jip.2004.10.004

Hwang, S.H., Saitoh, H., Mizuki, E., Higuchi, K., Ohba, M., 1998. A novel class of mosquitocidal δ-endotoxin, Cry19B, encoded by a *Bacillus thuringiensis* serovar *higo* gene. Syst. Appl. Microbiol. 21, 179–184. https://doi.org/10.1016/S0723-2020(98)80022-2

Ibrahim, M.A., Griko, N., Junker, M., Bulla, L.A., 2010. *Bacillus thuringiensis* A genomics and proteomics perspective. Bioeng. Bugs 1, 31–50. https://doi.org/10.4161/bbug.1.1.10519

Iriarte, J., Bel, Y., Ferrandis, M.D., Andrew, R., Murillo, J., Ferré, J., Caballero, P., 1998. Environmental distribution and diversity of *Bacillus thuringiensis* in Spain. Syst. Appl. Microbiol. 21, 97–106. https://doi.org/10.1016/S0723-2020(98)80012-X

Ishiwata, S., 1905. Concerning "Sotto-Kin" a Bacillus of a disease of the silkworm. Rept Assoc Seric Japan 160, 1–8.

Ishiwata, S., 1901. On a new type of severe flacherie (soto disease) (original in Japanese). Dainihon Sansi Kaiho 114, 1–5.

Ito, T., Bando, H., Asano, S. ichiro, 2006a. Activation process of the mosquitocidal δ-endotoxin Cry39A produced by

Bacillus thuringiensis subsp. *aizawai* BUN1-14 and binding property to *Anopheles stephensi* BBMV. J. Invertebr. Pathol. 93, 29–35. https://doi.org/10.1016/j.jip.2006.05.007

Ito, T., Ikeya, T., Sahara, K., Bando, H., Asano, S.I., 2006b. Cloning and expression of two crystal protein genes, cry30Ba1 and cry44Aa1, obtained from a highly mosquitocidal strain, *Bacillus thuringiensis* subsp. *entomocidus* INA288. Appl. Environ. Microbiol. 72, 5673–5676. https://doi.org/10.1128/AEM.01894-05

Ito, T., Sahara, K., Bando, H., Asano, S., 2002. Cloning and expression of novel crystal protein genes cry39A and 39orf2 from *Bacillus thuringiensis* subsp. *aizawai* Bun1-14 encoding mosquitocidal proteins. J. Insect Biotechnol. Sericology 71, 123–128.

Jenkins, J.L., Dean, D.H., 2000. Exploring the Mechanism of Action of Insecticidal Proteins by Genetic Engineering Methods, in: Plenum Press (Ed.), Genetic Engineering: Principles and Methods. New York, p. 33.

Johnson, C., Bishop, A.H., Turner, C.L., 1998. Isolation and Activity of Strains of *Bacillus thuringiensis* Toxic to Larvae of the Housefly (Diptera: Muscidae) and Tropical Blowflies (Diptera: Calliphoridae). J. Invertebr. Pathol. 71, 138–144. https://doi.org/10.1006/jipa.1997.4720

Jones, G.W., Nielsen-Leroux, C., Yang, Y., Yuan, Z., Dumas, V.F., Monnerat, R.G., Berry, C., 2007. A new Cry toxin with a unique two-component dependency from *Bacillus sphaericus*. FASEB J. 21, 4112–4120. https://doi.org/10.1096/fj.07-8913com

Jones, G.W., Wirth, M.C., Monnerat, R.G., Berry, C., 2008. The Cry48Aa-Cry49Aa binary toxin from *Bacillus sphaericus* exhibits highly restricted target specificity. Environ. Microbiol. 10, 2418–2424. https://doi.org/10.1111/j.1462-2920.2008.01667.x

Jouzani, G.S., Valijanian, E., Sharafi, R., 2017. *Bacillus thuringiensis*: a successful insecticide with new environmental features and tidings. Appl. Microbiol. Biotechnol. 101, 2691–2711. https://doi.org/10.1007/s00253-017-8175-y

Juárez-Pérez, V., Guerchicoff, A., Rubinstein, C., Delecluse, A., 2002. Characterization of Cyt2Bc Toxin from *Bacillus thuringiensis* subsp. *medellin*. Appl. Environ. Microbiol. 68, 1228–1231. https://doi.org/10.1128/AEM.68.3.1228

Juárez-pérez, V., Porcar, M., Orduz, S., Delécluse, A., 2003. Cry29A and Cry30A Two Novel delta-endotoxins Isolated from *Bacillus thuringiensis* serovar *medellin***.** Syst App Microbiol. 504, 502–504.

Jurat-Fuentes, J.L., Jackson, T.A., 2012. Bacterial entomopathogens, Insect Pathology. https://doi.org/10.1016/B978-0-12-384984-7.00008-7

Kamareddine, L., 2012. The biological control of the malaria vector. Toxins (Basel). 4, 748–767. https://doi.org/10.3390/toxins4090748

Kaur, S., 2000. Molecular approaches towards development of novel *Bacillus thuringiensis* biopesticides. World J. Microbiol. Biotechnol. 16, 781–793. https://doi.org/10.1023/A:1008931207374

Kawalek, M.D., 1998. Cloning and characterization of mosquitocidal genes from *Bacillus thuringiensis* subs. *jegathesan*. University of California, Riverside.

Knowles, B.H., Blatt, M.R., Tester, M., Horsnell, J.M., Carroll, J., Menestrina, G., Ellar, D.J., 1989. A cytolytic δ-endotoxin from *Bacillus thuringiensis* var. *israelensis* forms cation-selective channels in planar lipid bilayers. FEBS Lett. 244, 259–262. https://doi.org/10.1016/0014-5793(89)80540-X

Knowles, B.H., Dow, J. a. T., 1993. The Target : Introduction to the Lepidopteran Gut. BioEssays 15, 469–476.

Knowles, B.H., Ellar, D.J., 1987. Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificity. BBA - Gen. Subj. 924, 509–518. https://doi.org/10.1016/0304-4165(87)90167-X

Kongsuwan, K., Gough, J., Kemp, D., McDevitt, A., Akhurst, R., 2005. Characterization of a new *Bacillus thuringiensis* endotoxin, Cry47Aa, from strains that are toxic to the Australian sheep blowfly, *Lucilia cuprina*. FEMS Microbiol. Lett. 252, 127–136. https://doi.org/10.1016/j.femsle.2005.08.037

Koni, P.A., Ellar, D.J., 1994. Biochemical characterization of *Bacillus thuringiensis* cytolytic d-endotoxins. Microbiology 140, 1869–1880.

Kronstad, J.W., Schnepf, H.E., Whiteley, H.R., 1983. Diversity of locations for *Bacillus thuringiensis* crystal protein genes. J. Bacteriol. 154, 419–428.

Kumar, S., Chandra, A., Pandey, K., 2008. *Bacillus thuringiensis* (Bt) transgenic crop: An environment friendly insect-pest management strategy. Environ. Biol. J. 29, 641–653.

Kurtak, D., Jamnback, H., Meyer, R., Ocran, M., Renaud, P., 1987. Evaluation of larvicides for the control of Simulium damnosum s.l. (Diptera: Simuliidae) in West Africa. J. Mosq. Control Assoc. 3, 201–10.

Lacey, L., 1986. Microbial Control of Black Flies and Mosquitoes. Annu. Rev. Entomol. 31, 265–296. https://doi.org/10.1146/annurev.ento.31.1.265

Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: Back to the future. J. Invertebr. Pathol. 132, 1–41. https://doi.org/10.1016/j.jip.2015.07.009

Lacey, L.A., Smittle, B.J., 1985. The effects of gamma radiation on espore viability and mosquito larvicidal activity of *Bacillus sphaericus* and *Bacillus thuringiensis* var. *israelensis*. Bull. Soc. Vector Ecol. 10, 98–101.

Lecadet, M.M., Frachon, E., Cosmao Dumanoir, V., Ripouteau, H., Hamon, S., Laurent, P., Thiéry, I., 1999. Updating the H-antigen classification of *Bacillus thuringiensis*. J. Appl. Microbiol. 86, 660–672. https://doi.org/10.1046/j.1365-2672.1999.00710.x

Lee, H., Gill, S.S., 1997. Molecular cloning and characterization of a novel mosquitocidal protein gene from *Bacillus Molecular* Cloning and Characterization of a Novel Mosquitocidal Protein Gene from *Bacillus thuringiensis* subsp . *fukuokaensis* 63, 4664–4670.

Lee, H.K., Gill, S.S., 1997. Molecular cloning and characterization of a novel mosquitocidal protein gene from *Bacillus thuringiensis* subsp. *fukuokaensis*. Appl. Environ. Microbiol. 63, 4664–4670.

Lee, S.G., Eckblad, W., Lee, A.B., 1985. Diversity of Protein Inclusion Bodies and Identification of Mosquitocidal Protein in *Bacillus thuringiensis* subsp. *israelensis*. Biochem. Biophys. Res. Commun. 126, 953–960.

Lereclus, D., Delecluse, A., Lecadet, M.M., 1993. Diversity of *Bacillus thuringiensis* toxins and genes, in: Sons, J.W.& (Ed.), *Bacillus thuringiensis*, an Environmental Biopesticide: Theory and Practice. New York, pp. 37–69.

Li, J., Koni, P.A., Ellar, D.J., 1996. Structure of the mosquitocidal δ-endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. J. Mol. Biol. 257, 129–152. https://doi.org/10.1006/jmbi.1996.0152

Liang, H., Liu, Y., Zhu, J., Guan, P., Li, S., Wang, S., Zheng, A., Liu, H., Li, P., 2011. Characterization of Cry2-type genes of *Bacillus thuringiensis* strains from soil-isolated of sichuan basin, China. Brazilian J. Microbiol. 42, 140–146.

Likitvivatanavong, S., Gill, S.S., Bravo, A., Soberón, M., 2011. Cadherin, Alkaline Phosphatase, and Aminopeptidase N as Receptors of Cry11Ba Toxin from *Bacillus thuringiensis* subsp. *jegathesan* in *Aedes aegypti*. Appl. Environ. Microbiol. 77, 24–31. https://doi.org/10.1128/aem.01852-10

Liu, C., Lu, J., Lu, L., Liu, Y., Wang, F., Xiao, M., 2010. Isolation, structural characterization and immunological activity of an exopolysaccharide produced by Bacillus licheniformis 8-37-0-1. Bioresour. Technol. 101, 5528–5533. https://doi.org/10.1016/j.biortech.2010.01.151

Liu, J.W., Porter, A.G., Wee, B.Y., Thanabalu, T., 1996. New gene from nine *Bacillus sphaericus* strains encoding highly conserved 35.8-kilodalton mosquitocidal toxins. Appl. Environ. Microbiol. 62, 2174–2176.

López-Diaz, J.A., Cantón, P.E., Gill, S.S., Soberón, M., Bravo, A., 2013. Oligomerization is a key step in Cyt1Aa membrane insertion and toxicity but not necessary to synergize Cry11Aa toxicity in *Aedes aegypti* larvae. Environ. Microbiol. 15, 3030–3039. https://doi.org/10.1111/1462-2920.12263

Mac Innes, T.C., Bouwer, G., 2009. An improved bioassay for the detection of *Bacillus thuringiensis* β-exotoxin. J. Invertebr. Pathol. 101, 137–139. https://doi.org/10.1016/j.jip.2009.03.007

Manasherob, R., Ben-Dov, E., Xiaoqiang, W., Boussiba, S., Zaritsky, A., 2002. Protection from UV-B damage of mosquito larvicidal toxins from *Bacillus thuringiensis* subsp. *israelensis* expressed in Anabaena. PCC 7120. Curr. Microbiol. 45, 217–220. https://doi.org/10.1007/s00284-001-0106-5

Manasherob, R., Itsko, M., Sela-Baranes, N., Ben-Dov, E., Berry, C., Cohen, S., Zaritsky, A., 2006. Cyt1 Ca from *Bacillus thuringiensis* subsp. *israelensis*: Production in *Escherichia coli* and comparison of its biological activities with those of other Cyt-like proteins. Microbiology 152, 2651–2659. https://doi.org/10.1099/mic.0.28981-0

Manceva, S.D., Pusztai-Carey, M., Russo, P.S., Butko, P., 2005. A detergent-like mechanism of action of the cytolytic toxin Cyt1A from *Bacillus thuringiensis* var. *israelensis*. Biochemistry 44, 589–597. https://doi.org/10.1021/bi048493y

Marco, G., Porcar, M., 2012. Ecological Mysteries: is *Bacillus thuringiensis* a Real Insect Pathogen? Bt Res. 3, 1–2. https://doi.org/10.5376/bt.2012.03.0001

Margalith, Y., Ben-Dov, E., 2000. Insect pest management: techniques for environmental protection., CRC Press. ed.

Martin, P.A.W., Travers, R.S., 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol. 55, 2437–2442.

McClintock, J.T., Schaffer, C.R., Sjoblad, R.D., 1995. A comparative review of the mammalian toxicity of *Bacillus thuringiensis*-based pesticides. Pestic. Sci. 45, 95–105. https://doi.org/10.1002/ps.2780450202

Misra, H.S., Hire, R.S., Mahajan, S.K., 2002. Cloning and characterization of an insecticidal crystal protein gene from *Bacillus thuringiensis* subspecies *kenyae*. J. Genet. 81, 5–11. https://doi.org/10.1007/bf02715864

Moar, W.J., Trumble, J.T., Hice, R.H., Backman, P.A., 1994. Insecticidal activity of the CryIIA protein from the NRD-12 isolate of *Bacillus thuringiensis* subsp. *kurstaki* expressed in *Escherichia coli* and *Bacillus thuringiensis* and in a leaf-colonizing strain of *Bacillus cereus*. Appl. Environ. Microbiol. 60, 896–902.

Mulligan, F.S., Schaefer, C.H., Wilder, W.H., 1980. Efficacy and Persistence of *Bacillus sphaericus* and *B. thuringiensis* H. 14 Against Mosquitoes Under Laboratory and Field Conditions1. J. Econ. Entomol. 73, 684–688. https://doi.org/10.1093/jee/73.5.684

Nicholls, C.N., Ahmad, W., Ellar, D.J., 1989. Evidence for two different types of insecticidal P2 toxins with dual specificity in *Bacillus thuringiensis* subspecies. J. Bacteriol. 171, 5141–5147. https://doi.org/10.1128/jb.171.9.5141-5147.1989

Nicolas, L., Nielsen-Leroux, C., Charles, J.F., Delécluse, A., 1993. Respective role of the 42- and 51-kDa components of the *Bacillus sphaericus* toxin overexpressed in *Bacillus thuringiensis*. FEMS Microbiol. Lett. 106, 275–279. https://doi.org/10.1111/j.1574-6968.1993.tb05976.x

Nisnevitch, M., Cohen, S., Ben-Dov, E., Zaritsky, A., Sofer, Y., Cahan, R., 2006. Cyt2Ba of *Bacillus thuringiensis israelensis:* Activation by putative endogenous protease. Biochem. Biophys. Res. Commun. 99–105.

Oestergaard, J., Ehlers, R.U., Martínez-Ramírez, A.C., Real, M.D., 2007. Binding of Cyt1Aa and Cry11Aa toxins of *Bacillus thuringiensis* serovar *israelensis* to brush border membrane vesicles of *Tipula paludosa* (Diptera: Nematocera) and subsequent pore formation. Appl. Environ. Microbiol. 73, 3623–3629. https://doi.org/10.1128/AEM.01056-06

Omolo, E.O., James, M.D., Osir, E.O., Thomson, J.A., 1997. Cloning and expression of a *Bacillus thuringiensis* (L1-2) gene encoding a crystal protein active against *Glossina morsitans morsitans* and *Chilo partellus*. Curr. Microbiol. 34, 118–121. https://doi.org/10.1007/s002849900154

Orduz, S., Diaz, T., Restrepo, N., Patiño, M.M., Tamayo, M.C., 1996. Biochemical, Immunological and Toxicological Characteristics of the Crystal Proteins of *Bacillus thuringiensis* subsp. *medellin*. Mem. Inst. Oswaldo Cruz. 91, 231–237. https://doi.org/10.1590/S0074-02761996000200020

Orduz, S., Realpe, M., Arango, R., Murillo, L.A., Delecluse, A., 1998. Sequence of the cry11Bb11 gene from *Bacillus thuringiensis* subsp. *medellin* and toxicity analysis of its encoded protein. Biochim. Biophys. Acta 54, 267–272.

Otieno-Ayayo, Z.N., Zaritsky, A., Wirth, M.C., Manasherob, R., Khasdan, V., Cahan, R., Ben-Dov, E., 2008. Variations in the mosquito larvicidal activities of toxins from *Bacillus thuringiensis* ssp. *israelensis*. Environ. Microbiol. 10, 2191–2199. https://doi.org/10.1111/j.1462-2920.2008.01696.x

Palma, L., Muñoz, D., Berry, C., Murillo, J., Caballero, P., Caballero, P., 2014. *Bacillus thuringiensis* toxins: An overview of their biocidal activity. Toxins (Basel). 6, 3296–3325. https://doi.org/10.3390/toxins6123296

Pardo-López, L., Soberón, M., Bravo, A., 2013. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: Mode of action, insect resistance and consequences for crop protection. FEMS Microbiol. Rev. 37, 3–22. https://doi.org/10.1111/j.1574-6976.2012.00341.x

Park, H.-W., Bideshi, D.K., Federici, B.A., 2010. Properties and applied use of the mosquitocidal bacterium, *Bacillus sphaericus*. J. Asia Pac Entomol 13, 159–168. https://doi.org/10.1016/j.aspen.2010.03.002

Park, H.W., Bideshi, D.K., Johnson, J.J., Federici, B.A., 1999. Differential enhancement of Cry2A versus Cry11A yields in *Bacillus thuringiensis* by use of the cry3A STAB mRNA sequence. FEMS Microbiol. Lett. 181, 319–327. https://doi.org/10.1016/S0378-1097(99)00555-8

Partridge, M. R., Berry, C., 2002. Insecticidal activity of the Bacillus sphaericus Mtx1 toxin against Chironomus riparus. J. Invertebr. Pathol. 79, 135-136.

Payne, J., Kenneth, E., Navra, Kendrick, A., Uyeda, Christine, J.F., Stalder, Tracy, 1995. *Bacillus thuringiensis* isolate PS201T6. 5436002.

Pérez, C., Fernández, L.E., Gill, S.S., Segovia, L., Bravo, A., Rodríguez, M.H., Soberón, M., 2005. Cry11Aa toxin from *Bacillus thuringiensis* binds its receptor in *Aedes aegypti* mosquito larvae through loop α-8 of domain II . FEBS Lett. 579, 3508–3514. https://doi.org/10.1016/j.febslet.2005.05.032

Pérez, C., Muñoz-Garay, C., Portugal, L.C., Sánchez, J., Gill, S.S., Soberón, M., Bravo, A., 2007. *Bacillus thuringiensis* ssp. *israelensis* Cyt1Aa enhances activity of Cry11Aa toxin by facilitating the formation of a pre-pore oligomeric structure. Cell. Microbiol. 9, 2931–2937. https://doi.org/10.1111/j.1462-5822.2007.01007.x

Poncet, S., Bernard, C., Dervyn, E., Cayley, J., Klier, A., Rapoport, G., 1997. Improvement of *Bacillus sphaericus* toxicity against dipteran larvae by integration, via homologous recombination, of the Cry11A toxin gene from *Bacillus thuringiensis* subsp. *israelensis.* Appl. Environ. Microbiol. 63, 4413–4420.

Poncet, Sandrine, Delecluse, A., Klier, A., Rapoport, G., 1995. Evaluation of Synergistic Interactions among CryIVA, CryIVB, and CryIVD Toxic Components of *B. thuringiensis* subsp. *israelensis* Crystals. J. Invertebr. Pathol. 66, 131–135.

Poncet, S., Delecluse, A., Klier, A., Rapoport, G., 1995. Evaluation of Synergistic Interactions among the CryIVA, CryIVB, and CryIVD Toxic Components of *B. thuringiensis* subsp. *israelensis* Crystals. J. Invertebr. Pathol. 66, 131–135.

Promdonkoy, B., Chewawiwat, N., Tanapongpipat, S., Luxananil, P., Panyim, S., 2003. Cloning and characterization of a cytolytic and mosquito larvicidal δ-endotoxin from *Bacillus thuringiensis* subsp. *darmstadiensis*. Curr. Microbiol. 46, 94–98. https://doi.org/10.1007/s00284-002-3823-5

Promdonkoy, B., Ellar, D.J., 2003. Investigation of the pore-forming mechanism of a cytolytic δ-endotoxin from *Bacillus thuringiensis*. Biochem. J. 374, 255–259. https://doi.org/10.1042/bj20030437

Promdonkoy, B., Ellar, D.J., 2000. Membrane pore architecture of a cytolytic toxin from *Bacillus thuringiensis*. Biochem. J. 350, 275–282. https://doi.org/10.1042/0264-6021:3500275

Promdonkoy, B., Promdonkoy, P., Panyim, S., 2005. Co-expression of *Bacillus thuringiensis* Cry4Ba and Cyt2Aa2 in *Escherichia coli* revealed high synergism against *Aedes aegypti* and *Culex quinquefasciatus* larvae. FEMS Microbiol. Lett. 252, 121–126. https://doi.org/10.1016/j.femsle.2005.08.038

Raymond, B., Johnston, P.R., Nielsen-LeRoux, C., Lereclus, D., Crickmore, N., 2010. *Bacillus thuringiensis*: An

impotent pathogen? Trends Microbiol. 18, 189–194. https://doi.org/10.1016/j.tim.2010.02.006

Raymond, B., Lijek, R.S., Griffiths, R.I., Bonsall, M.B., 2008. Ecological consequences of ingestion of *Bacillus cereus* on *Bacillus thuringiensis* infections and on the gut flora of a lepidopteran host. J. Invertebr. Pathol. 99, 103–111. https://doi.org/10.1016/j.jip.2008.04.007

Restrepo, N., Gutierrez, D., Patiño, M.M., Thiéry, I., Delécluse, A., Orduz, S., 1997. Cloning, Expression and Toxicity of a Mosquitocidal Toxin Gene of *Bacillus thuringiensis* subsp. *medellin*. Mem. Inst. Oswaldo Cruz 92, 257–262. https://doi.org/10.1590/S0074-02761997000200021

Revina, L.P., Kostina, L.I., Ganushkina, L.A., Mikhailova, A.L., Zalunin, I.A., Chestukhina, G.G., 2004. Reconstruction of *Bacillus thuringiensis* ssp. *israelensis* Cry11A endotoxin from fragments corresponding to its N- and C-moieties restores its original biological activity. Biochem. 69, 181–187. https://doi.org/10.1023/B:BIRY.0000018949.70836.dc

Ricoldi, M. cabral, Soares Figueiredo, C., Apparecida Desiderio, J., 2018. Toxicity of Cry2 proteins from *Bacillus thuringiensis* subsp. *thuringiensis* strain T01-328 against *Aedes aegypti* (Diptera: Culicidae). Toxicology 85, 1–7. https://doi.org/10.1590/1808

Rodriguez-Almazan, C., Ruiz De Escudero, I., Cantón, P.E., Muñoz-Garay, C., Pérez, C., Gill, S.S., Soberón, M., Bravo, A., 2011. The amino- and carboxyl-terminal fragments of the *Bacillus thuringensis* Cyt1Aa toxin have differential roles in toxin oligomerization and pore formation. Biochemistry 50, 388–396. https://doi.org/10.1021/bi101239r

Roh, jong yul, Choi, jae young, Li, ming shun, Jin, byung Rae, 2007. *Bacillus thuringiensis* as a Specific, Safe, and Effective Tool for Insect Pest Control. J Microbiol. Biotechnol. 17, 547–559.

Rosso, M.L., Delécluse, A., 1997. Contribution of the 65-kilodalton protein encoded by the cloned gene cry19A to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *jegathesan.* Appl. Environ. Microbiol. 63, 4449–4455.

Saitoh, H., Hwang, S.H., Park, Y.S., Higuchi, K., Mizuki, E., Ohba, M., 2000. Cloning and characterization of a *Bacillus thuringiensis* serovar *higo* gene encoding a novel class of the δ-endotoxin protein, Cry27A, specifically active on the Anopheles mosquito. Syst. Appl. Microbiol. 23, 25–30. https://doi.org/10.1016/S0723-2020(00)80042-9

Saleem, F., Shakoori, A.R., 2010. Characterization of cry2A-type Gene(s) from Pakistani Isolates of *Bacillus thuringiensis* toxic to lepidopteran and dipteran Insects. Pak. J. Zool. 42, 181–193.

Salehi Jouzani, G., Pourjan Abad, A., Seifinejad, A., Marzban, R., Kariman, K., Maleki, B., 2008. Distribution and diversity of Dipteran-specific cry and cyt genes in native *Bacillus thuringiensis* strains obtained from different ecosystems of Iran. J. Ind. Microbiol. Biotechnol. 35, 83–94. https://doi.org/10.1007/s10295-007-0269-6

Sattar, S., Maiti, M.K., 2011. Molecular characterization of a novel vegetative insecticidal protein from *Bacillus thuringiensis* effective against sap-sucking insect pest. J. Microbiol. Biotechnol. 21, 937–946. https://doi.org/10.4014/jmb.1105.05030

Schmidt, T.R., Scott, E.J., Dyer, D.W., 2011. Whole-genome phylogenies of the family Bacillaceae and expansion of the sigma factor gene family in the *Bacillus cereus* species-group. BMC Genomics 12. https://doi.org/10.1186/1471-2164-12-430

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H., 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62, 775–806.

Sevim, A., Eryüzlü, E., Demirba, Z., Demir, I., 2012. A novel cry2Ab gene from the indigenous isolate *Bacillus thuringiensis* subsp. *kurstaki*. J. Microbiol. Biotechnol. 22, 133–140. https://doi.org/10.4014/jmb.1108.08061

Sims, S.R., 1997. Host activity spectrum of the CryIIA *Bacillus thuringiensis* subsp. *kurstaki* protein: effects on Lepidoptera, Diptera, and non-target arthropods. Southwest. Entomol.

Skovmand, O., Bauduin, S., 1998. Efficacy of a granular formulation of *Bacillus sphaericus* against *Culex quinquefasciatus* and *Anopheles gambiae* in West African countries. J. Vector Ecol. 43–51.

Smith, G.P., Merrick, J.D., Bone, E.J., Ellar, D.J., 1996. Mosquitocidal activity of the CryIC δ-endotoxin from *Bacillus thuringiensis* subsp. *aizawai*. Appl. Environ. Microbiol. 62, 680–684.

Soberón, M., López-Díaz, J.A., Bravo, A., 2013. Cyt toxins produced by *Bacillus thuringiensis*: A protein fold conserved in several pathogenic microorganisms. Peptides 41, 87–93. https://doi.org/10.1016/j.peptides.2012.05.023

Sun, Y., Zhao, Q., Xia, L., Ding, X., Hu, Q., Federici, B.A., Park, H.-W., 2013. Identification and Characterization of Three Previously Undescribed Crystal Proteins from *Bacillus thuringiensis* subsp. *jegathesan* . Appl. Environ. Microbiol. 79, 3364–3370. https://doi.org/10.1128/aem.00078-13

Tabashnik, B.E., 2008. Delaying insect resistance to transgenic crops. Proc. Natl. Acad. Sci. U. S. A. 105, 19029–19030. https://doi.org/10.1073/pnas.0810763106

Tabashnik, B.E., Schwartz, J.M., Finson, N., Johnson, M.W., 1990. Inheritance of Resistance to *Bacillus thuringiensis* in Diamondback Moth (Lepidoptera: Plutellidae). J. Econ. Entomol 85, 1046–1055.

Tan, F., Zheng, A., Zhu, J., Wang, L., Li, S., Deng, Q., Wang, S., Li, P., Tang, X., 2010. Rapid cloning, identification, and application of one novel crystal protein gene cry30Fa1 from *Bacillus thuringiensis*. FEMS Microbiol. Lett. 302, 46–51. https://doi.org/10.1111/j.1574-6968.2009.01829.x

Tan, F., Zhu, J., Tang, J., Tang, X., Wang, S., Zheng, A., Li, P., 2009. Cloning and characterization of two novel crystal protein genes, cry54Aa1 and cry30Fa1, from *Bacillus thuringiensis* strain BtMC28. Curr. Microbiol. 58, 654–659. https://doi.org/10.1007/s00284-009-9386-y

Thanabalu, T., Hindley, J., Jackson-Yap, J., Berry, C., 1991. Cloning, sequencing, and expression of a gene encoding a 100-kilodalton mosquitocidal toxin from *Bacillus sphaericus* SSII-1. J. Bacteriol. 173, 2776–2785. https://doi.org/10.1128/jb.173.9.2776-2785.1991

Thanabalu, T., Porter, A.G., 1996. A *Bacillus sphaericus* gene encoding a novel type of mosquitocidal toxin of 31.8 kDa. Gene 170, 85–89. https://doi.org/0378-1119/96/$15.00

Thiery, I., Delécluse, A., Tamayo, M.C., Orduz, S., 1997. Identification of a gene for Cyt1A-like hemolysin from *Bacillus thuringiensis* subsp. *medellin* and expression in a crystal-negative *B. thuringiensis* Strain. Appl. Environ. Microbiol. 63, 468–473.

Thiéry, I., Hamon, S., Delécluse, A., Orduz, S., 1998. The introduction into *Bacillus sphaericus* of the *Bacillus thuringiensis* subsp. *medellin* cyt1Ab1 gene results in higher susceptibility of resistant mosquito larva populations to *B. sphaericus*. Appl. Environ. Microbiol. 64, 3910–3916.

Thomas, W.E., Ellar, D.J., 1983. *Bacillus thuringiensis* var *israelensis* crystal delta-endotoxin: effects on insect and mammalian cells in vitro and in vivo. J. Cell Sci. 60, 181–197.

Thorne, L., Garduno, F., Thompson, T., Decker, D., Zounes, M., Wild, M., Waldfield, A., Pollock, T.J., 1986. Structural similarity between the Lepidoptera- and Diptera-specific insecticidal endotoxin genes of *Bacillus thuringiensis* subsp. "*kurstaki*" and "*israelensis*." J. Bacteriol. 166, 801–811. https://doi.org/10.1128/jb.166.3.801-811.1986

Torres-Quintero, M.C., Gómez, I., Pacheco, S., Sánchez, J., Flores, H., Osuna, J., Mendoza, G., Soberón, M., Bravo, A., 2018. Engineering *Bacillus thuringiensis* Cyt1Aa toxin specificity from dipteran to lepidopteran toxicity. Sci. Rep. 8, 1–12. https://doi.org/10.1038/s41598-018-22740-9

Vasquez, M.I., Violaris, M., Hadjivassilis, A., Wirth, M.C., 2009. Susceptibility of *Culex pipiens* (Diptera: Culicidae) Field Populations in Cyprus to Conventional Organic Insecticides, *Bacillus thuringiensis* subsp. *israelensis*, and Methoprene. J. Med. Entomol. 46, 881–887. https://doi.org/10.1603/033.046.0421

Vidal-Quist, J.C., Castañera, P., González-Cabrera, J., 2010. Cyt1Aa protein from *Bacillus thuringiensis* (Berliner) serovar *israelensis* is active against the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). Pest Manag. Sci. 66, 949–955. https://doi.org/10.1002/ps.1965

Waalwijk, C., Dullemans, A., Wiegers, G., Smits, P., 1992. Toxicity of *Bacillus thuringiensis* variety *israelensis* against tipulid larvae. J. Appl. Entomol. 114, 415–420. https://doi.org/10.1111/j.1439-0418.1992.tb01146.x

Ward, E.S., Ridley, A.R., Ellar, D.J., Todd, J.A., 1986. *Bacillus thuringiensis* var. *israelensis* δ-endotoxin. Cloning and expression of the toxin in sporogenic and asporogenic strains of *Bacillus subtilis*. J. Mol. Biol. 191, 13–22. https://doi.org/10.1016/0022-2836(86)90418-3

Wei, J.-Z., Hale, K., Carta, L., Platzer, E., Wong, C., Fang, S.-C., Aroian, R., 1993. *Bacillus thuringiensis* crystal proteins that target nematodes. Am. J. Hypertens. 5, 480–483. https://doi.org/10.1093/ajh/5.7.480

Whalon, M.E., Wingerd, B.A., 2003. Bt: Mode of action and use. Arch. Insect Biochem. Physiol. 54, 200–211. https://doi.org/10.1002/arch.10117

Widner, W.R., Whiteley, H.R., 1989. Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* possess different host range specificities. J. Bacteriol. 171, 965–974. https://doi.org/10.1128/jb.171.2.965-974.1989

Wirth, M.C., 2010. Mosquito Resistance to Bacterial Larvicidal Toxins. Open Toxinology J. 3, 101–115.

Wirth, M.C., Berry, C., Walton, W.E., Federici, B.A., 2014. Mtx toxins from *Lysinibacillus sphaericus* enhance mosquitocidal cry-toxin activity and suppress cry-resistance in *Culex quinquefasciatus*. J. Invertebr. Pathol. 115, 62–67. https://doi.org/10.1016/j.jip.2013.10.003

Wirth, M.C., Delecluse, A., Walton, W.E., 2001. Cyt1Ab1 and Cyt2Ba1 from *Bacillus thuringiensis* subsp. *medellin* and *B. thuringiensis* subsp. *israelensis* Synergize *Bacillus sphaericus* against *Aedes aegypti* and Resistant *Culex quinquefasciatus* (Diptera: Culicidae). Appl. Environ. Microbiol. 67, 3280–3284. https://doi.org/10.1128/AEM.67.7.3280

Wirth, M.C., Georghiou, G.P., 1997. Cross-Resistance Among CryIV Toxins of *Bacillus thuringiensis* subsp. *israelensis* in *Culex quinquefasciatus*. J. Econ. Entomol. 90, 1471.

Wirth, M.C., Georghiou, G.P., Malik, J.I., Abro, G.H., 2000. Laboratory Selection for Resistance to *Bacillus sphaericus* in *Culex quinquefasciatus* (Diptera: Culicidae) from California, USA . J. Med. Entomol. 37, 534–540. https://doi.org/10.1603/0022-2585-37.4.534

Wirth, M.C., Park, H.W., Walton, W.E., Federici, B.A., 2005. Cyt1A of *Bacillus thuringiensis* delays evolution of resistance to Cry11A in the mosquito *Culex quinquefasciatus*. Appl. Environ. Microbiol. 71, 185–189. https://doi.org/10.1128/AEM.71.1.185-189.2005

Wu, D., Federici, B.A., 1995. Improved production of the insecticidal CryIVD protein in *Bacillus thuringiensis* using cryIA(c) promoters to express the gene for an associated 20 kDa protein. Appl. Microbiol. Biotechnol. 42, 697–702. https://doi.org/10.1007/s002530050316

Wu, D., Johnson, J.J., Federici, B.A., 1994. Synergism of mosquitocidal toxicity between CytA and CrylVD proteins using inclusions produced from cloned genes of *Bacillus thuringiensis*. Mol. Microbiol. 13, 965–972. https://doi.org/10.1111/j.1365-2958.1994.tb00488.x

Xu, D., Côté, J.C., 2008. Sequence diversity of *Bacillus thuringiensis* flagellin (H antigen) protein at the intra-H serotype level. Appl. Environ. Microbiol. 74, 5524–5532. https://doi.org/10.1128/AEM.00951-08

Yamagiwa, M., Sakagawa, K., Sakai, H., 2004. Functional analysis of two processed fragments of *Bacillus thuringiensis* Cry11A toxin. Biosci. Biotechnol. Biochem. 68, 523–528. https://doi.org/10.1271/bbb.68.523

Yu, X., Liu, T., Sun, Z., Guan, P., Zhu, J., Wang, S., Li, S., Deng, Q., Wang, L., Zheng, A., Li, P., 2012. Co-expression and synergism analysis of Vip3Aa29 and Cyt2Aa3 insecticidal proteins from *Bacillus thuringiensis*. Curr. Microbiol. 64, 326–331. https://doi.org/10.1007/s00284-011-0070-7

Zhang, L., Zhao, G., Hu, X., Liu, J., Li, M., Batool, K., Chen, M., Wang, J., Xu, J., Huang, T., Pan, X., Xu, L., Yu, X.Q., Guan, X., 2017. Cry11Aa Interacts with the ATP-Binding Protein from *Culex quinquefasciatus* to Improve the Toxicity. J. Agric. Food Chem. 65, 10884–10890. https://doi.org/10.1021/acs.jafc.7b04427

Zhang, W., Yu, S., Peng, S., Gong, J., Qian, J., He, J., Dai, W., Wang, R., 2017. Characterization of a novel mosquitocidal toxin of Cry50Ba and its potential synergism with other mosquitocidal toxins. Toxicon 138, 165–168. https://doi.org/10.1016/j.toxicon.2017.08.025

Zhong, C., Ellar, D.J., Bishop, A., Johnson, C., Lin, S., Hart, E.R., 2000. Characterization of a *Bacillus thuringiensis* δ-endotoxin which is toxic to insects in three orders. J. Invertebr. Pathol. 76, 131–139. https://doi.org/10.1006/jipa.2000.4962

Zhong, C., Peng, D., Ye, W., Chai, L., Qi, J., Yu, Z., Ruan, L., Sun, M., 2011. Determination of plasmid copy number reveals the total plasmid DNA amount is greater than the chromosomal DNA amount in *Bacillus thuringiensis* YBT-1520. PLoS One 6. https://doi.org/10.1371/journal.pone.0016025

Zhu, J, Zheng, A., Wang, S., Liu, H., Li, P., 2010. Characterization and expression of cry4Cb1 and cry30Ga1 from *Bacillus thuringiensis* strain HS18-1. J. Invertebr. Pathol. 103, 200–202. https://doi.org/10.1016/j.jip.2009.12.004

Zhu, J., Zheng, A.P., Tan, F.R., Wang, S.Q., Deng, Q.M., Li, S.C., Wang, L.X., Li, P., 2010. Characterisation and expression of a novel holotype crystal protein gene, cry56Aa1, from *Bacillus thuringiensis* strain Ywc2-8. Biotechnol. Lett. 32, 283–288. https://doi.org/10.1007/s10529-009-0147-6

CHAPTER II

Quantification of dose-mortality responses in adult Diptera: validation using *Ceratitis capitata* and *Drosophila suzukii* responses to spinosad

Abstract

Quantitative laboratory bioassay methods are required to evaluate the toxicity of novel insecticidal compounds for pest control and to determine the presence of resistance traits. We used a radioactive tracer based on ^{32}P-ATP to estimate the volume of a droplet ingested by two dipteran pests: *Ceratitis capitata* (Tephritidae) and *Drosophila suzukii* (Drosophilidae). Using a blue dye it was possible to distinguish between individuals that ingested the solution from those that did not. The average (±SE) volume ingested by *C. capitata* adults was 1.968 ± 0.049 µl. Females ingested a ~20% greater volume of solution than males ($P<0.001$). Adults of *D. suzukii* ingested an average of 0.879 ± 0.035 µl and females ingested ~30% greater volume than males ($P<0.001$). The droplet feeding method was validated using the naturally-derived insecticide spinosad as the active ingredient (a.i.). For *C. capitata*, the concentration-mortality response did not differ significantly between the sexes ($P>0.05$) or among three different batches of insects. Lethal dose values were calculated based on mean ingested volumes. For *C. capitata* LD_{50} values were 1.462 and 1.502 ng a.i./insect for males and females, respectively, equivalent to 0.274 and 0.271 ng a.i./mg for males and females, respectively, when sex-specific variation in body weight was considered. Using the same process for *D. suzukii*, the LD_{50} value was estimated at 2.927 ng a.i./insect, or 1.994 ng a.i./mg based on a mean body weight of 1.67 ± 0.09 mg for both sexes together. We conclude that this technique could be readily employed for determination of the resistance status and dose-mortality responses of insecticidal compounds in many species of pestiferous Diptera.

This chapter has been published to PlosOne as: **Daniel Valtierra-de-Luis, Maite Villanueva, Javier Caballero, Isabel M. Matas, Trevor Williams, Primitivo Caballero. 2019.** Quantification of dose-mortality responses in adult Diptera: validation using *Ceratitis capitata* and *Drosophila suzukii* responses to spinosad.

TABLE OF CONTENTS

1. INTRODUCTION

Simple and repeatable laboratory bioassay methods are required to evaluate the toxicity of novel insecticidal compounds for pest management and to determine the presence of resistance traits in pest populations (Robertson et al., 2017). The most suitable bioassay methods usually reflect the mode of action of the toxicant and the biology of the pest. Established assay methods therefore use direct topical application, spray droplet delivery systems, such as the Potter tower, or contact with residues on treated surfaces to establish concentration-mortality responses of pests under controlled laboratory conditions (Brodsgaard, 1994; Crowder et al., 1979). These methods have proved to be valuable for compounds that are absorbed following contact with the insect cuticle, such as organophosphates or pyrethroids (Longley and Stark, 1996; Penman et al., 1981; Scott, 1990). However, many of the latest generation of insecticidal products that have a selective toxicity spectrum are active by ingestion. Examples include naturally-derived products such as spinosad, avermectins, and *Bacillus thuringiensis* (Bt), and synthetic compounds such as neonicotinoids and diamides, among others (Nauen et al., 2012).

The accurate quantification of toxicity requires the ability to deliver a known quantity of toxicant onto, or into, the pest. For leaf-feeding pests this can be accomplished by placing known quantities of toxicant on leaf discs or on pieces of artificial diet that, when consumed, deliver a known dose of toxicant. For flies however, precise delivery of toxicants by ingestion is more challenging as many feed on liquids that are taken into the crop before being gradually released into the midgut for digestion and assimilation (Stoffolano and Haselton, 2013). In the case of dipterans, quantification of the concentration-mortality response requires a method for distinguishing flies that have fed on the toxicant solution from those that have not. This can be achieved by mixing the toxicant with an inert food coloring agent that can be visualized through the abdomen of flies that consumed the colored liquid (Baudier et al., 2014). In contrast, dose-mortality studies require quantification of the volume of toxicant solution ingested by experimental flies.

In the present study we demonstrate how the use of food-dye based differentiation of toxicant-treated individuals and quantification of ingested volume can be used to characterize the dose-mortality

relationship according to sex and body weight of two pestiferous invasive species of Diptera. These species were *Ceratitis capitata* (Wiedemann) (Tephritidae) and *Drosophila suzukii* (Matsumura) (Drosophilidae), both of which are recognized as pests that should be subjected to international quarantine regulations (EPPO 2019). *Ceratitis capitata* is a polyphagous pest affecting more than 250 species of fruits and vegetables (McPheron et al 1996). This fly can survive across a wide range of hosts and climatic conditions and has become established in the Mediterranean region, Africa, the Middle East, Latin America and Western Australia (EPPO 2019). *Drosophila suzukii* is an invasive pest endemic to south-east Asia that has recently established in most European countries, North America, and the Middle East. This pest attacks a wide range of both cultivated and wild soft-skinned fruits, particularly berries (Cini et al., 2012). The larval stages of both these species feed deep within fruit, protected from external applications of pesticides. Consequently, control strategies targeted at these pests usually focus on the adult stage through the use of traps, toxic bait stations, or insecticides applied to host plant foliage, where adults may be resting or feeding, either through catholic foliar or bait sprays (Epsky et al., 1999; Lasa et al., 2017; Navarro-Llopis et al., 2013; Van Timmeren and Isaacs, 2013), although the use of the sterile insect technique may be effective in some regions (Dyck et al., 2006).

In the present study we use a radioactive tracer to estimate the volume ingested by flies of each sex and species and perform a proof-of-concept study using the naturally-derived insecticide, spinosad to determine concentration-mortality, dose-mortality and dose per mg body weight relationships. Spinosad was selected for this study as it is highly active by ingestion and has proven to be effective in the control of fruit flies in many parts of the world (Adan et al., 1996; Burns et al., 2001).

2. MATERIALS AND METHODS

2.1.Insect colonies

Pupae of *C. capitata* were obtained at weekly intervals from a colony maintained at the Universidad Politécnica de Madrid, Spain. These insects had been reared on an artificial diet as described previously (Zapata et al., 2006), and had no history of exposure to insecticides. Following emergence, adults were held in ventilated plastic cages 11 x 9 x 8.5 cm with continuous access to water and a

mixture of sucrose and hydrolyzed brewer's yeast (4:1) as food. A laboratory colony of *D. suzukii* was started using pupae obtained from the Institut de Recerca i Tecnologia Agroalimentàries [IRTA], Barcelona, Spain. The colony was maintained in the Instituto de Agrobiotecnología, Mutilva, Spain, on a solid semi-synthetic diet comprising 10.5g/l agar, 60 g/l brewer's yeast, 50 g/l sucrose, 10 g/l ground soybean, 60 g/l maize flour, 10 ml/l ethanol, 5 ml/l propionic acid and 20 mM methyl-paraben. Adult flies were maintained in 14 l ventilated plastic containers. Adults had continuous access to water and the same diet as used to rear larvae. Adult flies of both species and all experimental procedures described in the following sections were performed under the same laboratory conditions of 24 ± 1 °C, 85 ± 10% relative humidity and a 16 h: 8 h (light: dark) photoperiod.

2.2. Determination of ingested volume by adult flies

Groups of 50 flies of both sexes that had emerged in the previous 24 h period were placed in 300 ml plastic cups with a muslin lid. Flies were starved, without access to food or water, for 12 h and were then given access to 5 µl droplets of a solution comprising radiolabeled adenosine triphosphate (ATP, γ-^{32}P, 3000Ci/mmol, PerkinElmer), 0.1 mg/ml fluorella blue (Hilton Davis, USA), 0.5% (wt./vol.) hydrolyzed protein (Attrack, Cheminova Agro, Spain), 15% (wt./vol.) sucrose. Flies were allowed to consume the liquid during a 20 min period and were then immediately placed in a -20°C freezer for 1 h until completely frozen. The individuals that had consumed the radioactive liquid were identified by the blue coloration of the intestine observed through the abdominal wall. These individuals were sexed and placed individually in a plastic tube (MicroBeta Trilux 4ml counting vials, PerkinElmer) with 1.5 ml Ultima Gold liquid scintillation cocktail (PerkinElmer). Adult flies with unstained or partially-stained abdomens were discarded. The radioactivity of each intact individual was determined over a 60 second period using a scintillation counter (MicroBeta 1450 Trilux Wallac, Perkin Elmer, USA). Each fly was measured five times and the average value was calculated after correction for background radiation. The average ingested volume value was determined by comparing the average number of counts obtained from each fly with a calibration curve previously determined using a range of dilutions of ATP-γ-^{32}P (Supplemental material, S1 Fig). The experiment was performed using a total of 112 *C. capitata* adults (57 female, 55 male) and 64 *D. suzukii* adults (42 female, 22 male).

2.3. Body weight of flies

To determine the body weight of flies, adults that had emerged 12-24 h previously and had not mated, were placed in a 300 ml plastic cup and starved for 12 h. Cups were then placed in an Anaerocult A anaerobic jar (Merck, Germany) with dry ice to generate an anaerobic atmosphere that anaesthetized flies. After 15 minutes, each fly was weighed individually to a precision of 0.1 mg using an electronic balance (Ohaus Pioneer, USA). A total of 40 *C. capitata* and 30 *D. suzukii* individuals of each sex were weighed.

2.4. Validation of method using spinosad

As a proof-of-concept, toxicity assays were performed on both species of flies. For bioassays with *C. capitata*, 15-20 adults were collected in 300 ml plastic cups sealed with a muslin lid and starved for 12 h. Flies were then offered 5 µl droplets placed on a piece of parafilm for 20 min (30 droplets in total, ~5 mm distance between droplets). Experimental droplets contained 0.1 mg/ml fluorella blue, 0.5% (wt./vol.) hydrolyzed protein (Attrack 300, Cheminova Agro, Spain), 15% (wt./vol.) sucrose and spinosad as the active ingredient (a.i.). The concentration-mortality response was determined in groups of 15-20 adults of both sexes that consumed one of six different concentrations of spinosad (Spintor 480 SC, Dow AgroScience, Spain) from 0.2 to 1.5 µg a.i./ml (previously estimated to kill between 5 and 95% of experimental insects). In the case of *C. capitata* males, the range of concentrations was 0.3 to 1.5 µg a.i./ml. Droplets of an identical solution containing distilled water only were offered to insects as a control. After 20 min, the parafilm strip with droplets was removed and adult flies that did not have fully blue-colored abdomens were removed and discarded, leaving approximately 20 flies in each 300 ml cup. A 25 ml plastic cup containing a small piece of sponge soaked with 20 ml of a liquid diet comprising 30% sucrose, 0.5% Attrack and 0.05% methyl-paraben as preservative, was then placed into the larger cup. Mortality was recorded at 5 days post-treatment, by which time LC_{50} values had plateaued according to a previous study (Adan et al., 1996) Supplemental material S2 Fig). A total of four batches of *C. capitata* were tested. The assay was replicated four times for batches 1 and 2 and three times for batches 3 and 4. Males and females were assayed separately in batch 4.

Bioassays with *D. suzukii* adults were performed using a similar design except that droplets of spinosad solution were 3 µl rather than 5 µl in volume and adult diet contained 5% (wt./vol.) soy peptone (Conda, Spain) instead of 0.5% Attrack. The concentration-mortality response was determined using six concentrations of spinosad ranging from 1.39 µg/ml to 5.2 µg/ml. This assay was performed on three occasions using three different groups of insects (replicates) from the laboratory colony.

2.5. Statistical analyses

Ingested volumes and body weights of adult flies of each sex were compared by Welch's unequal variances t-test. Concentration-mortality data were subjected to logit regression to estimate the median lethal concentration (LC_{50}). Abbott's correction was applied to mortality data prior to analysis to correct for low levels of control mortality (Abbot 1925). The significance of treatment and interaction terms was determined by sequential removal of terms from the complete logit regression model. All statistical procedures were performed using R software (v. 3.5.1).

3. RESULTS

3.1. Determination of ingested volume by adult flies

Using the blue dye in the ingested experimental solution it was possible to differentiate individuals of *C. capitata* and *D. suzukii* that ingested the solution from those that did not ingest it, or those that partially ingested it (Fig 1, A and B). Insects that did not consume the solution in the 20 min feeding period were discarded.

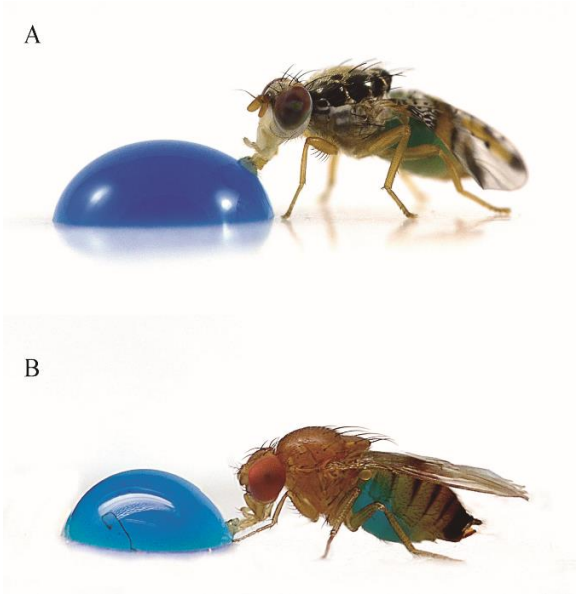


Figure 4. Adults of (A) *Ceratitis capitata* and (B) *Drosophila suzukii* that fed on experimental droplets could be identified by the blue coloration of their abdomen.

The average (±SE) volume of solution ingested by *C. capitata* adults was 1.968 ± 0.049 µl (Table 1), but this differed significantly between sexes with females ingesting ~20% greater volume of solution than males (Welch t = 4.96, d.f. = 94.8, P<0.001). In contrast, adults of *D. suzukii* ingested an average of 0.879 ± 0.035 µl and females ingested ~30% greater volume than males (Welch t = 4.69, d.f. = 50.07, P<0.001) (Table 1, S1 Fig.).

Table 1. Mean ingested volume (±SE) per individual for each sex separately and both sexes of *Ceratitis capitata* and *Drosophila suzukii* adults.

Species	Sex	Number of individuals	Ingested volume ± SE (µl/fly)
	Both sexes	112	1.968 ± 0.049
C. capitata	Male	55	1.741 ± 0.075
	Female	57	2.187 ± 0.050
	Both sexes	64	0.879 ± 0.035
D. suzukii	Male	22	0.692 ± 0.046
	Female	42	0.977 0.040

3.2. Body weight of flies

The average (±SE) live weight of individual *C. capitata* was 5.43 ± 0.06 mg. The average weight of males (5.33 ± 0.09 mg) was not significantly different from that of females (5.54 ± 0.09 mg) (Welch t = 1.61, d.f. = 79.78, P = 0.110). In contrast, the average live weight of *D. suzukii* adults was 1.67 ± 0.09 mg and females (2.063 ± 0.055 mg) were significantly heavier than males (1.30 ± 0.05 mg) (Welch t = 10.61, d.f. = 56.87, P<0.001).

3.3. Validation of method using spinosad

Mortality in control groups of insects ranged from 0 - 4% and this was used to adjust observed mortality using Abbott's correction (Abbott, 1925). In all cases, mortality increased with increasing concentration of spinosad. For *C. capitata*, the LC_{50} values varied between 0.677 and 0.773 µg a.i./ml for both sexes together (Table 2). The concentration-mortality response was similar among batches of insects (batch: $\chi^2 = 1.06$, d.f. = 2, P = 0.588), although the slope of the response differed significantly among batches 1-3 (interaction batch*concentration: $\chi^2 = 25.7$, d.f. = 12, P < 0.001). When sexes were treated separately (batch 4), estimated LC_{50} values were 0.840 µg a.i./ml in females and 0.687 µg

a.i./ml in males, and the concentration-mortality response did not differ significantly between the sexes (sex: $F_{1,9} = 0.028$, P = 0.872; interaction sex*concentration: $F_{1,7} = 2.68$, P = 0.146) (Table 2).

When the mean volume of toxicant solution consumed by insects was considered, LD_{50} values for *C. capitata* varied from 1.332 to 1.521 ng a.i./insect for both sexes together, compared to 1.462 and 1.502 ng a.i./insect for males and females, respectively (Table 2). When the mean of body weight of *C. capitata* adults was taken into account, LD_{50} values per mg of insect body weight were 0.280, 0.261, 0.245 ng a.i./mg for both sexes together in batches 1 - 3, respectively, and were 0.274 and 0.271 ng a.i./mg for males and females in batch 4, respectively.

Using the same process, for *D. suzukii* the LC_{50} value was estimated at 3.330 µg a.i./ml and the LD_{50} value was estimated at 2.927 ng a.i./insect, based on the mean ingested volume of 0.879 ± 0.035 µl/insect (Table 1). Similarly, for *D. suzukii* the LD_{50} value per mg of live body weight was 1.994 ng a.i./mg based on a mean body weight of 1.67 ± 0.09 mg for both sexes together.

Table 2. Logit regression of concentration-mortality response of *Ceratitis capitata* and *Drosophila suzukii* adults that consumed a range of concentrations of spinosad. Bioassays were performed on three batches of *C. capitata* (both sexes) and an additional batch batch (4) in which each sex was treated separately.

Batch	Regression		LC_{50}	95% C.I.		LD_{50}	95% C.I.	
	Slope ± SE	Intercept ± SE	µg a.i. /ml	Lower	Upper	ng a.i. /insect	Lower	Upper
Ceratitis capitata								
1	2.84 ± 0.29	-2.19 ± 0.22	0.773	0.665	0.881	1.521	1.308	1.733
2	4.45 ± 0.40	-3.22 ± 0.28	0.723	0.640	0.806	1.423	1.259	1.586
3	5.72 ± 0.62	-3.87 ± 0.40	0.677	0.597	0.757	1.332	1.175	1.490
4 (♂)	4.05 ± 0.39	-3.39 ± 0.39	0.840	0.726	0.948	1.462	1.263	1.650
4 (♀)	5.17 ± 0.56	-3.55 ± 0.38	0.687	0.597	0.777	1.502	1.305	1.699
Drosophila suzukii								
1	1.42 ± 0.14	-4.72 ± 0.46	3.330	3.223	3.437	2.927	2.799	3.336

LD_{50} values were calculated based on LC_{50} values and the volume of solution ingested on average by each species (shown in Table 1)

4. DISCUSSION

Quantification of ingestion of a sucrose solution containing ^{32}P-labeled ATP revealed that *C. capitata* males and females ingested an average of 1.74 and 2.19 µl of solution respectively, whereas *D. suzukii* consumed 0.69 and 0.98 µl for each sex, respectively (Table 1). This information was used to estimate lethal dose response of both species to the naturally derived insecticide spinosad, from lethal concentration response studies. The 50% lethal dose (LD_{50}) of *C. capitata* to spinosad was approximately half that of *D. suzukii*, although in terms of live body weight, *C. capitata* was approximately 7-fold more susceptible to spinosad than *D. suzukii* (based on values of 0.27 and 1.84 ng a.i./mg body weight, respectively).

Spinosad has been used in bait formulations to control tephritid pests for almost two decades (Burns et al., 2001; Peck and McQuate, 2000), whereas its use against *D. suzukii* (Bruck et al., 2011; Shawer et al., 2018) has largely coincided with the global expansion of this pest and its introduction into North America and Europe over the past decade (Walsh et al., 2011). This compound has a highly favorable ecotoxicological profile and has a low impact on most insect natural enemy populations (Stark et al., 2004; Williams et al., 2003).

Previous estimates of spinosad toxicity to *C. capitata* have ranged from 0.896 mg a.i./l at 48 h post-treatment (Voudouris et al., 2017) to 0.24 - 0.28 mg a.i./l following five days of continuous exposure (Adan et al., 1996; Mura and Ruiu, 2018), whereas studies involving a different methodology reported significantly higher LC_{50} values of 2.8 and 4.2 mg a.i./l for males and females, respectively, at 24 h post-treatment (Stark et al., 2004). Individuals of *C. capitata* that survive exposure to sublethal quantities of spinosad can also experience negative effects on fecundity, egg fertility, and adult longevity, in addition to sex-dependent effects on the expression of a number of immune-modulating genes (Mura and Ruiu, 2018).

Previous studies on spinosad toxicity to *D. suzukii* reported that high concentrations resulted in 100% mortality, although concentration-mortality responses were not determined (Beers et al., 2011; Bruck et al., 2011; Cowles et al., 2015; Cuthbertson et al., 2014; Van Timmeren and Isaacs, 2013). One

exception involved a study on spinosad contact toxicity to two *D. suzukii* populations in which contact LC_{50} values of 2.78 and 7.60 mg a.i./l were estimated following 6 h of exposure (Mishra et al., 2017).

In addition to the dose of toxicant consumed, the influence of speed of kill of the compound can affect the apparent toxicity of a compound in laboratory assays, in which measures of mortality are taken shortly after exposure to the toxicant. In the present study, we adopted a 5-day period for assessment of spinosad-induced mortality, based on a previous study in which spinosad-induced cumulative mortality increased and estimated LC_{50} values plateaued over a 7-day period (Adan et al., 1996), S2 Fig.). This reflected the slower speed of kill of spinosad compared to fast acting compounds, such as pyrethroids. However, the 5-day period of post-treatment monitoring did not adversely affect the prevalence of mortality of untreated control insects that never exceeded 4% mortality in any case.

Females of both species consumed larger volumes of toxicant solution than conspecific males, although when lethal dose per mg of body weight was calculated, the susceptibility of both sexes to spinosad was similar in *C. capitata* (0.27 ng a.i./mg body weight for both sexes). In contrast, in *D. suzukii* the sexes were not treated separately as this would involve chilling or anaesthetizing them for manual sorting into sexes, which given their small size may have affected their survival during the post-treatment period.

Quantification of ingestion by dipterans is an issue that has received considerable attention, mainly because *D. melanogaster* has become a laboratory model for studies on physiology, nutrition and longevity for which precise estimates of feeding rates are required (Wong et al., 2009). The quantitative methods developed to date include the use of calibrated capillary feeding tubes (the CAFE system) (Ja et al., 2007), recording the proboscis extension feeding response (Mair et al., 2005; Wong et al., 2009), and the use of food in droplets mixed with dye (Edgecomb et al., 1994) or radioactive tracer compounds (Carvalho et al., 2005; Geer et al., 1970). Each of these methods has advantages and drawbacks, but for studies over short periods, the use of radiolabeled tracers in droplets of food has been shown to be highly sensitive, consistent and compatible with natural feeding behavior in *Drosophila* (Deshpande et al., 2014). This aspect of feeding on droplets is likely to extend to other dipterans such as tephritids that feed on droplets of honeydew, fruit juices or bird droppings in the

natural environment (J. Hendrichs and M. A. Hendrichs, 1990; Jácome et al., 1999; Manrakhan and Lux, 2006). From a logistical standpoint, once calibrated, the droplet-feeding assay is also quick and easy to set up and the presence of a visible dye in the mixture allows individuals that feed on toxicant droplets to be readily distinguished from those that did not. The food dye based methodology, either as is or slightly modified, could also be used to study the deterrence of fruit flies to ingestion of insecticides by assessing the prevalence of flies with unstained or partially-stained abdomens.

There are numerous sources of variation in insect bioassays involving ingestion of toxins or pathogens that have to be managed to control overdispersion in the results (Navon and Ascher, 2000; Robertson et al., 1995). In the case of dipterans, these include individual-level effects, such as sporadic or irregular feeding patterns over time, variation in meal size, and body size, developmental and physiological effects. Genetically distinct strains of insects are also expected to feed and respond differently to a given inoculum or toxicant (Kaun et al., 2007; Robertson et al., 1995), particularly if they have a history of exposure to a given compound (Jin et al., 2011). Moreover, as apparent in the present study, there is often sex-dependent variation in insect ingestion and sensitivity to toxicants. In the case of *D. melanogaster*, females that have mated also show a marked increase in feeding, probably in a response to the energetic requirements for egg development (Carvalho et al., 2006).

Much of the variation present in insect bioassays can be controlled by standardizing insect rearing conditions (diet, density, temperature, etc.), selecting individuals of the same age and developmental stage, limiting the time available for ingestion of the toxicant to avoid repeated feeding events and using visible dyes to identify individuals that have not engaged in feeding on toxicant droplets. These were precisely the steps that we adopted to minimize variation in our bioassays.

The period during which experimental droplets can be ingested, 20 min in the case of our study, is relevant as excretion begins approximately 40 mins after feeding in *Drosophila* (Wong et al., 2009), which would have adversely affected the quantification of ingested volumes in the radioactive tracer study. Fortunately, the steps that we took to standardize experimental insect selection and dosing procedures resulted in low levels of variation within assays and among assays involving different batches of insects in the case of *C. capitata* (Table 2). This type of droplet feeding assay can be readily

calibrated to quantify the susceptibility of other species of pestiferous Diptera to insecticidal compounds or pathogens that act by ingestion, such as *Bacillus thuringiensis* or some viruses (Cossentine et al., 2016; Gravot et al., 2000; Habayeb et al., 2009; Prompiboon et al., 2010; Vidal-Quist et al., 2009).

We conclude that the present study represents a quantitative method for determining the toxic properties of insecticidal compounds or pathogens that act by ingestion in adult Diptera that feed by consuming droplets. The method is simple and reproducible. To our knowledge, this is also the first report of quantification of lethal dose responses by ingestion of an insecticide and ingested lethal doses per mg of insect body weight in adult Diptera.

5. ACKNOWLEDGEMENTS

We thank Pilar Medina (Universidad Politécnica de Madrid) and Rosa Gabarra (Institut de Recerca i Tecnologia Agroalimentàries, Barcelona) for providing insects. Gabriel Mercado provided logistical support to TW.

6. REFERENCES

Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. J Econ Entomol. 18, 265–267. doi:10.1093/jee/18.2.265a

Adan, A., Del Estal, P., Budia, F., Gonzalez, M., Viñuela, E., 1996. Laboratory evaluation of the novel naturally derived compound spinosad against *Ceratitis capitata*. Pest Manag Sci. 48, 261-268.

Baudier, K.M., Kaschock-Marenda, S.D., Patel, N., Diangelus, K.L., O'Donnell, S., Marenda, D.R., 2014. Erythritol, a non-nutritive sugar alcohol sweetener and the main component of Truvia®, is a palatable ingested insecticide. PLoS ONE 9, e98949. doi:10.1371/journal.pone.0098949.s003

Beers, E.H., Van Steenwyk, R.A., Shearer, P.W., Coates, W.W., Grant, J.A., 2011. Developing *Drosophila suzukii* management programs for sweet cherry in the western United States. Pest. Manag. Sci. 67, 1386–1395. doi:10.1002/ps.2225

Brodsgaard, H.F., 1994. Insecticide Resistance in European and African strains of western flower thrips (Thysanoptera: Thripidae) tested in a new residue-on-glass test. J Econ Entomol. 87, 1141-1146.

Bruck, D.J., Bolda, M., Tanigoshi, L., Klick, J., Kleiber, J., DeFrancesco, J., Gerdeman, B., Spitler, H., 2011. Laboratory and field comparisons of insecticides to reduce infestation of *Drosophila suzukii* in berry crops. Pest. Manag. Sci. 67, 1375–1385. doi:10.1016/j.ibmb.2004.03.018

Burns, R.E., Harris, D.L., Moreno, D.S., Eger, J.E., 2001. Efficacy of spinosad bait sprays to control Mediterranean and Caribbean fruit flies. Florida Entomol. 84, 672-678.

Carvalho, G.B., Kapahi, P., Anderson, D.J., Benzer, S., 2006. Allocrine modulation of feeding behavior by the sex peptide of *Drosophila*. Current Biology 16, 692–696. doi:10.1016/j.cub.2006.02.064

Carvalho, G.B., Kapahi, P., Benzer, S., 2005. Compensatory ingestion upon dietary restriction in *Drosophila melanogaster*. Nat Chem Biol 2, 813–815. doi:10.1371/journal.pbio.0030223

Cini, A., Ioriatti, C., Anfora, G., 2012. A review of the invasion of *Drosophila suzukii* in Europe and a draft research agenda for integrated pest management. Bull insectol. 65, 149-160.

Cossentine, J., Robertson, M., Xu, D., 2016. Biological activity of *Bacillus thuringiensis* in *Drosophila suzukii* (Diptera: Drosophilidae). J Econ Entomol 109, 1071–1078. doi:10.1006/jipa.2000.4962

Cowles, R.S., Rodriguez-Saona, C., Holdcraft, R., Loeb, G.M., Elsensohn, J.E., Hesler, S.P., 2015. Sucrose improves insecticide activity against *Drosophila suzukii* (Diptera: Drosophilidae). J Econ Entomol. 108, 640–653. doi:10.1653/024.097.0212

Crowder, L.A., Tollefson, M.S., Watson, T.F., 1979. Dosage-mortality studies of synthetic pyrethroids and methyl parathion on the tobacco budworm in central Arizona. J Econ Entomol. 72, 1–3.

Cuthbertson, A., Blackburn, L., Audsley, N., 2014. Efficacy of commercially available invertebrate predators against *Drosophila suzukii*. Insects 5, 952–960. doi:10.1002/ps.3296

Deshpande, S.A., Carvalho, G.B., Amador, A., Phillips, A.M., Hoxha, S., Lizotte, K.J., Ja, W.W., 2014. Quantifying *Drosophila* food intake: comparative analysis of current methodology. Nat Chem Biol 11, 535–540. doi:10.1021/pr300613c

Dyck, V.A., Hendrichs, J., Robinson, A.S., 2006. Sterile insect technique: principles and practice in area-wide integrated pest management. Netherlands: Springer.

Edgecomb, R.S., Harth, C.E., Schneiderman, A.M., 1994. Regulation of feeding behavior in adult *Drosophila melanogaster* varies with feeding regime and nutritional state. J. Exp. Biol. 197, 215–235.

EPPO. [cited 2018 Dec 19]. European and Mediterranean Plant Protection Organization, A2 List of pests recommended for regulation as quarantine pests. Available from: http://www.eppo.int/ACTIVITIES/plant_quarentine/A2_list

Epsky, N.D., Hendrichs, J., Katsoyannos, B.I., Vasquez, L.A., Ros, J.P., Zumreoglu, A., Pereira, R., Bakri, A., Seewooruthun, S.I., Heath, R.R., 1999. Field evaluation of female-targeted trapping systems for *Ceratitis capitata* (Diptera: Tephritidae) in seven countries. 92: 156-164.

Geer, B.W., Olander, R.M., Sharp, P.L., 1970. Quantification of dietary choline utilization in adult *Drosophila melanogaster* by radioisotope methods. J. Insect Physiol. 16, 33-43.

Gravot, E., Thomas-Orillard, M., Jeune, B., 2000. Virulence variability of the *Drosophila* C virus and effects of the microparasite on demographic parameters of the host (*Drosophila melanogaster*). J Invertebr Pathol 75, 144–151. doi:10.1006/jipa.1999.4913

Habayeb, M.S., Cantera, R., Casanova, G., Ekström, J.-O., Albright, S., Hultmark, D., 2009. The Drosophila Nora virus is an enteric virus, transmitted via feces. J Invertebr Pathol 101, 29–33. doi:10.1016/j.jip.2009.02.003

Hendrichs, J., Hendrichs, M.A., 1990. Mediterranean fruit fly (Diptera: Tephritidae) in nature: location and diel pattern of feeding and other activities on fruiting and nonfruiting hosts and nonhosts. Ann. Entomol. Soc. Am. 83, 632-641.

Ja, W.W., Carvalho, G.B., Mak, E.M., la Rosa, de, N.N., Fang, A.Y., Liong, J.C., Brummel, T., Benzer, S., 2007. Prandiology of *Drosophila* and the CAFE assay. Proc. Natl. Acad. Sci. U.S.A. 104, 8253–8256. doi:10.1073/pnas.0702726104

Jácome, I., Aluja, M., Liedo, P., 1999. Impact of adult diet on demographic and population parameters of the tropical fruit fly *Anastrepha serpentina* (Diptera: Tephritidae). Bull. Entomol. Res. 89, 165-1275.

Jin, T., Zeng, L., Lin, Y., Lu, Y., Liang, G., 2011. Insecticide resistance of the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), in mainland China. Pest. Manag. Sci. 67, 370–376. doi:10.1002/ps.2076

Kaun, K.R., Riedl, C.A.L., Chakaborty-Chatterjee, M., Belay, A.T., Douglas, S.J., Gibbs, A.G., Sokolowski, M.B., 2007. Natural variation in food acquisition mediated via a *Drosophila* cGMP-dependent protein kinase. J. Exp. Biol. 210, 3547–3558. doi:10.1242/jeb.006924

Lasa, R., Tadeo, E., Toledo-Hérnandez, R.A., Carmona, L., Lima, I., Williams, T., 2017. Improved capture of *Drosophila suzukii* by a trap baited with two attractants in the same device. PLoS ONE 12, e0188350. doi:10.1371/journal.pone.0188350

Longley, M., Stark, J.D., 1996. Analytical techniques for quantifying direct, residual, and oral exposure of an insect parasitoid to an organophosphate insecticide. Bull Environ Contam Toxicol 57, 683–690.

Mair, W., Piper, M.D.W., Partridge, L., 2005. Calories do not explain extension of life span by dietary restriction in *Drosophila*. PLoS Biol 3, e223. doi:10.1371/journal.pbio.0030223.st001

Manrakhan, A., Lux, S.A., 2006. Contribution of natural food sources to reproductive behaviour, fecundity and longevity of *Ceratitis cosyra*, *C. fasciventris* and *C. capitata* (Diptera: Tephritidae). Bull. Entomol. Res. 96, 259–268. doi:10.1093/aesa/74.5.462

McPheron BA, Steck GJ eds. 1996. Fruit fly pests: a world assessment of their biology and management. Delray Beach, FL: St Lucie Press.

Mishra, R., Chiu, J.C., Hua, G., Tawari, N.R., Adang, M.J., Sial, A.A., 2017. High throughput sequencing reveals *Drosophila suzukii* responses to insecticides. Insect Sci 309, 764. doi:10.1002/(SICI)1096-9063(199904)55:4<398::AID-PS925>3.0.CO;2-O

Mura, M.E., Ruiu, L., 2018. Sex-specific sub-lethal effects and immune response in *Ceratitis capitata* Wied. (Diptera: Tephritidae) challenged with spinosad. Insects 9, 73. doi:10.3390/insects9030073

Nauen, R., Elbert, A., McCaffery, A., Slater, R., Sparks, T.C., 2012. IRAC: insecticide resistance, and mode of action classification of insecticides. In: Modern Crop Protection Compounds. 3, 935-956.

Navarro-Llopis, V., Primo, J., Vacas, S., 2013. Efficacy of attract-and-kill devices for the control of *Ceratitis capitata*. Pest. Manag. Sci. 69, 478–482. doi:10.1002/ps.3393

Navon, A., Ascher, K.R.S., 2000. Bioassays of entomophatogenic microbes and nematodes. CABI Publishing, Wallingford, UK.

Peck, S.L., McQuate, G.T., 2000. Field tests of environmentally friendly malathion replacements to suppress wild Mediterranean fruit fly (Diptera: Tephritidae) populations. J Econ Entomol 93, 280–289. doi:10.1603/0022-0493-93.2.280

Penman, D.R., Chapman, R.B., Jesson, K.E., 1981. Effects of fenvalerate and azinphosmethyl on two-spotted spider mite and phytoseiid mites. Ent. exp. and appl. 30, 91-97.

Prompiboon, P., Lietze, V.-U., Denton, J.S.S., Geden, C.J., Steenberg, T., Boucias, D.G., 2010. *Musca domestica* salivary gland hypertrophy virus, a globally distributed insect virus that infects and sterilizes female houseflies. Appl. Environ. Microbiol. 76, 994–998. doi:10.1128/AEM.02424-09

Robertson, J.L., Jones, M.M., Olguin, E., Alberts, B., 2017. Bioassays with arthropods. 3 edition. CRC press.

Robertson, J.L., Preisler, H.K., NG, S.S., Hickle, L.A., Gelernter, W.D., 1995. Natural Variation: A complicating factor in bioassays with chemical and microbial pesticides. J. Econ. Entomol. 88, 1–10.

Scott, J.G., 1990. Investigating mechanisms of insecticide resistance: methods, strategies, and pitfalls. In: Pesticide resistance in arthropods. Boston: Springer. 39-57.

Shawer, R., Tonina, L., Tirello, P., Duso, C., Mori, N., 2018. Laboratory and field trials to identify effective chemical control strategies for integrated management of *Drosophila suzukii* in European cherry orchards. Crop Protection 103, 73–80. doi:10.1016/j.cropro.2017.09.010

Stark, J.D., Vargas, R., Miller, N., 2004. Toxicity of spinosad in protein bait to three economically important tephritid fruit fly species (Diptera: Tephritidae) and their parasitoids (Hymenoptera: Braconidae). J Econ Entomol 97, 911–915.

Stoffolano, J.G., Haselton, A.T., 2013. The adult dipteran crop: a unique and overlooked organ. Annu. Rev. Entomol. 58, 205–225. doi:10.1146/annurev-ento-120811-153653

Van Timmeren, S., Isaacs, R., 2013. Control of spotted wing drosophila, *Drosophila suzukii*, by specific insecticides and by conventional and organic crop protection programs. Crop Protection 54, 126–133. doi:10.1016/j.cropro.2013.08.003

Vidal-Quist, J.C., Castañera, P., González-Cabrera, J., 2009. Diversity of *Bacillus thuringiensis* strains isolated from citrus orchards in Spain and evaluation of their insecticidal activity against *Ceratitis capitata*. J. Microbiol. Biotechnol. 19, 749–759. doi:10.4014/jmb.0810.595

Voudouris, C.C., Mavridis, K., Kalaitzaki, A., Skouras, P.J., Kati, A.N., Eliopoulos, P.A., Vontas, J., Margaritopoulos, J.T., 2017. Susceptibility of *Ceratitis capitata* to deltamethrin and spinosad in Greece. J Pest Sci 91, 861–871. doi:10.1007/s10340-017-0913-5

Walsh, D.B., Bolda, M.P., Goodhue, R.E., Dreves, A.J., Lee, J., Bruck, D.J., Walton, V.M., O'Neal, S.D., Zalom, F.G., 2011. *Drosophila suzukii* (Diptera: Drosophilidae): invasive pest of ripening soft fruit expanding its geographic range and damage potential. J Integr Pest Manag 2, G1–G7. doi:10.1603/IPM10010

Williams, T., Valle, J., Viñuela, E., 2003. Is the naturally derived insecticide Spinosad® compatible with insect natural enemies? Biocontrol Sci and Tech 13, 459–475. doi:10.1080/0958315031000140956

Wong, R., Piper, M.D.W., Wertheim, B., Partridge, L., 2009. Quantification of food intake in *Drosophila*. PLoS ONE 4, e6063. doi:10.1371/journal.pone.0006063

Zapata, N., Budia, F., Viñuela, E., Medina, P., 2006. Insecticidal effects of various concentrations of selected extractions of *Cestrum parqui* on adult and immature *Ceratitis capitata*. J Econ Entomol 99, 359–365.

CHAPTER III

Synergistic interaction of the δ-endotoxins Cry10Aa and Cyt2Ba from *Bacillus thuringiensis* ser. *israelensis* in *Aedes aegypti* larvae.

Abstract

Bacillus thuringiensis ser. *israelensis* (Bti) has been widely used as microbial larvicide for the control of many species of mosquitoes and blackflies. The larvicidal activity of Bti resides in Cry and Cyt δ-endotoxins present in the parasporal crystal of this entomopathogenic bacteria. The insecticidal activity of the crystal is high in comparison to the activities of the individual toxins, which is likely due to synergistic interactions among the crystal component proteins, particularly those involving Cyt1Aa. In the present study Cry10Aa and Cyt2Ba were cloned from the commercial product VectoBac and expressed in the acrystalliferous strain BMB171 under the *cyt1Aa* strong promoter of the pSTAB vector. These proteins had LC_{50} values of 299.62 and 279.37 ng/ml, respectively, against second instar *A. aegypti* larvae recording mortality at 24 hours. Marked synergism was detected between Cyt2B and Cry10Aa spore-crystal complexes ingested by the larvae in equal proportions. The magnitude of potentiation was 34.26, one of the highest described so far for Bti crystal components and comparable to that of Cyt1A with Cry4 and Cry11A. However, none of the proteins used in this work nor their mixture, produce a toxic effect against adults of *C.capitata*. We conclude that Bti minor components have relevance in the overall toxicity of the strain and both proteins can play an important role in synergy and resistance avoidance.

This chapter will be submitted to Pest Management Science as: **Valtierra-de-Luis, Daniel; Villanueva, Maite; Trevor Williams; and Caballero, Primitivo**. 2019. Synergistic interaction of the δ-endotoxins Cry10Aa and Cyt2Ba from *Bacillus thuringiensis* ser. *israelensis* in *Aedes aegypti* larvae.

TABLE OF CONTENT

1. INTRODUCTION

Bacillus thuringiensis ser. *israelensis* (Bti) was the first Bt serotype found to be toxic for dipteran species (Goldberg and Margalit, 1977). Bti forms parasporal inclusion bodies composed of insecticidal proteins (δ-endotoxins) that are widely used as the basis for microbial larvicides against several dipteran species, including mosquitoes, blackflies and chironomids (Federici et al., 2010; Margalith, Y., and Ben-Dov, 2000). Bti based products are considered to be powerful and highly selective larvicides for the control of disease vectors (Fillinger and Lindsay, 2006; Margalith, Y., and Ben-Dov, 2000). Indeed, Bti has been used to control mosquitoes for more than 35 years without any reports of resistance in vector populations (Becker and Ludwig, 1993; Vasquez et al., 2009). The absence of resistance is likely due to the varying modes of action and the synergistic effects of the multiple crystal proteins present in Bti-based products (Crickmore et al., 1995; Poncet et al., 1995; Wirth et al., 2005).

The parasporal crystal of Bti contains large amounts of four toxins: Cry4A, Cry4B, Cry11A and Cyt1A (Ibarra, J E., Federici, 1986). In addition, Cry10Aa and Cyt2Ba have also been described in some Bti strains although these are expressed and accumulated in the crystal in much smaller quantities that the four main components (Garduno et al., 1988; Lee et al., 1985). The Cry10Aa protein was cloned and named CryIVC, according to the existing classification at that time, but was described as a protein with a low larvicidal potency (Thorne et al., 1986). Later, the *cry10Aa* gene was identified as part of an operon that comprises two open reading frames (*orf1* and *orf2*) separated by a 66 bp gap (Berry et al., 2002). Cloning of the complete operon, linked to the strong promoter of the *cyt1A* gene, revealed that Cry10Aa was expressed at high levels and exhibited high larvicidal activity, both alone and in combination with Cyt1A. (Hernández-Soto et al., 2009). In contrast, although present at relatively low abundance in the Bti crystal (Guerchicoff et al., 1997), Cyt2Ba exhibits lower activity against *Aedes aegypti* larvae than the better-studied Cyt1Aa protein (Juárez-Pérez et al., 2002).

The interactions among the Cry and Cyt proteins of Bti have received more attention than any of the other Bt serovars (Crickmore et al., 1995; Hernández-Soto et al., 2009; Manasherob et al., 2006a; Otieno-Ayayo et al., 2008; Poncet et al., 1995; Wu et al., 1994). Interactions involving the Cyt1A protein have attracted particular attention given the capacity of this protein to enhance the insecticidal

activity of Cry proteins in strains of Bti (Crickmore et al., 1995; Hernández-Soto et al., 2009; Wu et al., 1994), and those of Bt strains belonging to other serovars (Wirth et al., 2000). Conversely, studies on the interactions of Cyt2Ba with other components of the Bti crystal are restricted to a single report of low synergistic activity of Cyt2Ba with the Cry4Aa protein (Manasherob et al., 2006b).

In the present study we want to analyse the implication of minor proteins in the whole Bti crystal toxicity. For that reason two recombinant Bt strains were constructed to express Cry10Aa and Cyt2Ba at high levels of expression. We provide evidence that both proteins interacted synergistically when simultaneously ingested by *Aedes aegypti* larvae. The result showed that Cyt2Ba is a synergistic factor that can be as important as Cyt1A to avoid the generation of resistances to Bti.

2. MATERIALS AND METHODS

2.1 Bacterial strains, plasmids and insect colonies.

The strains and plasmids used in this study are listed in Table 1. *B. thuringiensis* ser. *israelensis* (Bti) was isolated from VectoBac-12AS® (Kenogard, Spain). *Escherichia coli* XL1 blue was used for transformation. The acrystalliferous Bt strain BMB171 was used as the host strain for protein expression (Li et al., 2000). The recombinant vector pSTAB (Park et al., 1998) was used as the protein expression vector, engineered with the gene of interest. The Bt strains were grown in the CCY medium (13 mM KH_2PO_4, 26 mM K_2HPO_4, 10ml/l Nutrient Stock Solution (composed by L-glutamina, casein hydrolisate, bacto casitona. bacto yeast extract and glycerol), 1ml/l Metal Salts Solution (composed by several metal salts) (Stewart et al., 2001) at 28° C with continuous shaking at 200 rpm. All *E. coli* strains were cultured at 37 ºC with continuous shaking (200 rpm) in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0). When required for selective growth, LB medium was supplemented with 20 ng ml^{-1} erythromycin (Em) and 100 ng ml^{-1} ampicillin (Amp).

A laboratory colony of *A. aegypti* was started using eggs obtained from Susana Vilchez, (Universidad de Granada, Spain). The colony was maintained, under controlled environmental conditions (25 ±1 ºC, 85% RH, and a 16h :8h (light: dark) photoperiod, in the facilities of the Instituto Multidiciplinario de Biología Aplicada (IMBA, Pamplona, Spain). Adults of both sexes were maintained in BugDorm-1 insect rearing cages (MegaView Science, Taiwan) and had continuous access to 20% sucrose

solution and intermittent access (3h/day) to defibrinated horse blood (Thermo Scientific, UK) to complete their gonotrophic cycle. Larvae were reared in 250 ml glass beakers (40-50 larvae/glass) with 100ml distilled water and brewer's yeast (1mg/mL) as food (Lallemand, Canada).

Pupae of the tephritid fruit fly, *Ceratitis capitata* (Diptera: Tephritidae), were obtained at weekly intervals from a colony maintained at the Crop Protection Unit of the Universidad Politécnica de Madrid (Madrid, Spain). These insects had been reared on an artificial diet (Zapata et al., 2006), and had no history of exposure to insecticides. Following emergence, adults of both sexes were held in BugDorm-1 insects rearing cages with continuous access to water and a 4:1 mixture of sucrose and hydrolyzed brewer's yeast as food. Adult flies rearing and experimental procedures described in the following sections were performed under the same laboratory conditions of 24±1 ºC, 85±10% relative humidity and 16h: 8h (light:dark) photoperiod.

2.2 Total DNA extraction and genomic sequencing

Total genomic DNA (chromosomal + plasmid) was extracted following the protocol for DNA isolation from Gram-positive bacteria using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). A DNA library was prepared from total DNA and was subsequently sequenced in an llumina NextSeq500 Sequencer (Genomics Research Hub Laboratory, School of Biosciences, Cardiff University, UK).

2.3 Bioinformatics analyses

Genomic raw sequence data were processed and assembled using CLC Genomics Workbench 10.1.1. Reads were trimmed, filtered by low quality and reads of less than 50 bp were eliminated. Processed reads were assembled *de novo* using stringent criteria of at least 95 bp overlap and 95% identity. Reads were then mapped back to the contigs for assembly. Genes were predicted using GeneMark (Borodovsky and McIninch, 1993).

To assist in the identification of potential insecticidal proteins, local BLASTp (Altschul et al., 1990) was deployed against a database built in our laboratory comprising the amino acid sequences of known Bt toxins available at http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt (Crickmore et al., 1998), as well as other protein toxins of interest, including metalloproteinases and other mosquitocidal

toxins available in GenBank. PlasFlow software was used for prediction of plasmid sequences from the assembled contigs (Krawczyk et al., 2018).

2.4 Amplification and cloning of *cry10Aa* and *cyt2Ba* genes

Primers were designed to amplify the full-length coding sequence of *cyt2Ba* and the *cry10Aa* operon including *orf1* and *orf2*. Primer sequences included 5'-Xba and 3'-PstI restriction sites for *cyt2Ba*, as well as 5'-SalI and 3'-PaeI restriction sites for *cry10Aa*. PCR reactions were performed using Phusion DNA polymerase (NEB, USA) and amplicons were gel-purified using NucleoSpin Extract II kit (Macherey-Nagel, Germany). Purified products were then ligated into pJET1.2/blunt plasmid (CloneJET PCR Cloning Kit, Fermentas, Canada) following the manufacturer's instructions. Ligation mixtures were transformed into *E. coli* XL1-Blue using standard procedures (Sambrook and Russel, 2001). Colony-PCR was applied in order to check positive clones from which plasmid DNA was purified, using the NucleoSpin^{R} plasmid kit (Macherey-Nagel Inc., Bethlehem, PA) following the manufacturer's instructions. Subsequently, pJET plasmids were verified by sequencing (STABVida, Caparica, Portugal), digested with the appropriate combination of restriction enzymes, electrophoresed in 1% agarose gel and ligated into pre-digested pSTAB vector using the Rapid DNA ligation kit (Thermo Scientific) to obtain the recombinant plasmids pSTAB-*cyt2Ba* and pSTAB-*cry10Aa*. Ligation products were then electroporated into *E. coli* XL1 blue cells following standard protocols (Sambrook and Russell, 2001). Positive clones were verified by colony-PCR and plasmids were purified and verified by restriction endonuclease digestion and electrophoresis. Once pSTAB-*cyt2Ba* and pSTAB-*cry10Aa* were obtained, they were introduced into the acrystalliferous Bt strain BMB171.

Bacillus electrocompetent cells were generated as described previously (Dominguez-Arrizabalaga et al., 2019). Briefly, bacteria were grown in 300 ml of Brain Heart Infusion (BHI) commercial broth (Pronadisa) at 28°C under shaking conditions (200 rpm) until the culture reached an OD600 nm of 0.4. Glycine was then added to the culture at 2% and bacterial cells were incubated for another hour, at 28ºC under shaking conditions (200 rpm). Bacterial cells were kept on ice for 5 min, centrifuged at 9000 g (4 ºC) for 10 min and the pellet was washed three times with F buffer (272 mM sucrose, 0.5

mM $MgCl_2$, 0.5 mM K_2HPO_4, 0.5 mM KH_2PO_4, pH 7.2). Bacterial cells were then resuspended in 600 µl of ice-cold F buffer and stored in aliquots of 50 µl at -80°C. Plasmids were transformed into the BMB171 strain by electroporation, using a previously described protocol (Lee, 1995) . Positive clones were selected by colony-PCR. BMB171 was also transformed with an empty plasmid as a negative control.

Table 1. Sequences of PCR and sequencing primers.

Primer name	Primer sequence
Cyt2B-Fw-XbaI	5'-TTCTAGAGATAATGAAGGAGGGGAGTC-3'
Cyt2B-Rv-PstI	5'-CCTGCAGCAAAATTAAATTGCTGAGTTACTATAATAAC-3'
Cry10A-Fw-SalI	5'-ATGTCGACTTGCAACAGAAAAGAGTTGTGTC-3'
Cry10A-Rv-PaeI	5'-GAGCATGCACATTTCCCCACAATTTTCA-3'
Cry10A-test-Fw	5'-CGAAATTGTCAGACATAGAGAG-3'
Cry10A-test-Rv	5'-GAATTACCAAGTCTCCACCTG-3'

Restriction enzymes are underlined

2.5 Expression of Cry10Aa and Cyt2Ba recombinant proteins and SDS-PAGE analysis

Recombinant Bt strains were grown at 28ºC, under shaking conditions (200rpm), in CCY medium supplemented with 20 ng ml^{-1} erythromycin. Crystal formation was observed daily under the optical microscope. After 2-3 days, when ~95% of the cells had lysed, the mixture of spores and crystals was collected by centrifugation at 10,000×*g*, for 10 min at 4ºC. The pellet was washed once with saline solution (1M NaCl, 10 mM EDTA) and three times with 10 mM KCl. The spore + crystal mixture was finally resuspended in 10 mM KCl and kept at 4 ºC until used. Samples of spores and crystals were mixed with 2x sample buffer (Bio-Rad), boiled at 100 °C for 5 min, and then subjected to electrophoresis as previously described (Laemmli, 1970), using Criterion TGX™ 4-20% Precast Gel (BIO-RAD). Gels were stained with Coomassie brilliant blue R-250 (Bio-Rad) and then distained in 30% ethanol and 10% acetic acid. For protein quantification a 10uL aliquot of spores and crystals was solubilized *in vitro* in 1 mL of an alkaline solution (50mM Na_2CO_3 10 mM DTT, pH 11.3) for 2 hours

at 37 ºC. The protein concentration of each preparation was measured by the Bradford assay (Bradford, 1976) (Bio-Rad), using bovine serum albumin (BSA) as a standard. Bacterial protoxins were solubilized by suspension of 100 ul spores-crystals mixtures in 900 ul of solubilisation buffer (50mM Na_2CO_3 10 mM DTT, pH 11.3), incubated for 2 hours at 37 ºC with gentle agitation and centrifugated at 9000g for 15 min at 4 ºC. The protein concentration of the solubilised protoxins was determined according to the Bradford assay described above.

2.6 Toxicity bioassays

The toxicity of individual proteins and mixtures of Cry10Aa and Cyt2Ba was determined by bioassay against *A. aegypti* second instar larvae. Concentration-mortality bioassays were performed following a modified method described by McLaughlin (1984). Toxin concentrations were 2000, 666, 222, 74, 24.7 and 8.2 ng/ml for Cry10Aa and 4000, 1333, 444, 148, 49.4 and 16 ng/ml for Cyt2Ba. A mixture of Cry10Aa and Cyt2Ba in equal proportions was tested at concentrations of $3x10^{-1}$, $6x10^{-2}$, $1.2x10^{-2}$, $2.4x10^{-3}$, $4.8x10^{-4}$ and $9.6x10^{-5}$ ng/ml. Groups of 10 - 15 second instar larvae were tested with each concentration of Bt alone or in mixtures. Each group of larvae was placed in one well of a 6-well cell culture plate (Costar) containing 5 ml of a solution with the corresponding toxin concentration and 0.5 mg of brewer's yeast as food. Control insects were mock-infected. Each bioassay was performed at least three times. Insects were incubated at 25 ºC and 16:8 (L:D) photoperiod. Mortality was recorded at 24 h post-treatment. The toxicity of individual proteins and mixtures of crystalized and solubilized Cry10Aa and Cyt2Ba against *C. capitata* adults was determined by the bioassay method described by Valtierra et al 2019 (Chapter II).

2.7 Statistical analysis

Concentration-mortality data were subjected to Logit regression to estimate the median lethal concentration (LC_{50}) for individual toxins and the mixture of toxins. The significance of treatment and interaction terms was determined by sequential removal of terms from the complete regression model. The observed and expected LC_{50} values for the individual toxins and the toxin mixture in *A. aegypti* were used to evaluate the interaction of Cry10Aa and Cyt2Ba. To calculate the expected LC_{50} values for the toxin mixture under the null hypothesis of no interaction the “simple similar action” model was

used (Tabashnik, 1992). This model assumes that concentration-response regression lines for different components of a mixture are parallel and is suitable for testing synergism in chemically similar compounds such as Bt toxins.

The expected LC_{50} was calculated as follows:

$$LC50(m) = \left[\frac{\mathrm{rA}}{\mathrm{LC50(A)}} + \frac{\mathrm{rB}}{\mathrm{LC50(B)}}\right]^{-1}$$

Where $LC_{50(m)}$ is the expected LC_{50} of the mixture of toxin A and toxin B, $LC_{50(A)}$ is the observed LC_{50} for toxin A alone, $LC_{50(B)}$ is the observed LC_{50} for toxin B alone and r_A and r_B represent the relative proportions of toxin A and toxin B in the mixture, respectively. All statistical procedures were performed using R software (v.3.5.1).

3. RESULTS

3.1 Characterization of *cry10Aa* and *cyt2Ba*

BMB171 recombinant strains expressing Cry10Aa and Cyt2Ba were observed daily by phase-contrast microscopy and both recombinants sporulated normally in CCY medium. In parallel to sporulation, the formation of parasporal crystals was observed in both recombinant strains. Strain BMB171-Cry10Aa produced amorphous semi-spherical shaped crystals that were as big as the spore, and which were located at the opposite end of the cell to that occupied by the endospore (Figure 1A). In contrast, the crystals produced by the BMB171-Cyt2Ba strain were irregular and smaller than the spore (Figure 1B). As expected, vegetative cells of BMB171 strain transformed with an empty plasmid produced endospores but no crystals.

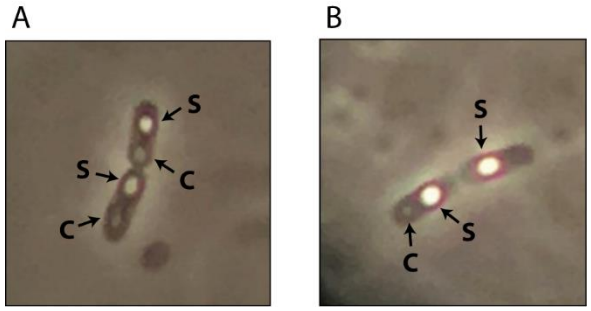


Figure 1. Optical microscope images of the following recombinant BMB171 strains: (A) BMB171-Cry10Aa; (B) BMB171-Cyt2Ba. Arrow labeled C indicates parasporal crystal, whereas arrow labeled S indicates presence of endospore.

SDS-PAGE showed that the recombinant BMB171-Cry10Aa expressed two proteins with molecular masses of approximately 68 and 56 kDa, which correspond to the predicted sizes of the proteins encoded by *orf1* and *orf2*, respectively, of the *cry10Aa* operon (Figure 2, lane 2). Similarly, the sample of spores and crystals produced by the recombinant strain BMB171-Cyt2Ba was characterized by a band of approximately 29 kDa, which corresponds to the size of the Cyt2Ba protein (Figure 2, lane 3).

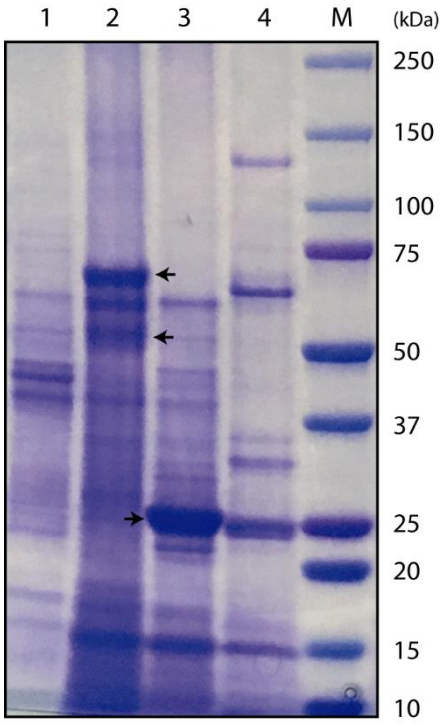


Figure 2. SDS-PAGE gel showing the protein profiles of the recombinant BMB171 strains and the Bti strain present in VectoBac. Lane 1, BMB171 acrystalliferous strain with an empty plasmid; lane 2, BMB171-Cry10Aa; lane 3, BMB171-Cyt2Ba; lane 4, wild-type Bti strain from VectoBac; Lane M, molecular mass marker.

3.2 Mosquitocidal activity

Both recombinant proteins exhibited a high insecticidal activity against *A. aegypti* second instars when ingested individually. LC_{50} values were estimated at 299.62 ng/ml and 279.37 ng/ml for Cry10Aa and Cyt2Ba, respectively and the slopes of the regressions did not differ significantly (interaction treatment*concentration: F=0.6202, d.f.=8, P=0.454). When treatments were analyzed all together (Cry10Aa+Cyt2Ba+Mixture), the slopes of the regression differed significantly (interaction treatment*concentration: F=7.359, d.f.=12, P=0.00821) due to the mixture treatment (the slope of the mixture regression line is higher). Thus fulfilling the principal assumption of the simple similar action model. The estimated LC_{50} value of the 1:1 mixture of Cry10Aa and Cyt2Ba was 4.22 ng/ml, compared to the expected value of LC_{50} of 144.58 ng/ml, assuming additive action of each of the toxins (Tabashnik 1992). Ingestion of the proteins Cry10Aa and Cyt2Ba in mixture of equal proportions increased the toxicity of the toxins by a factor of 34.26 (Table 2). The BMB171 strain with the empty plasmid showed no mortality. None of the strains or their mixture showed toxicity against *C. capitata* adult flies, either as crystal-spore preparations or as solubilized preparations.

Table 2. Logit analysis of concentration-mortality results of different Bti proteins for *Aedes aegypti* second instars at 24 h post-inoculation.

Treatment[a]	Regression		LC_{50} Observed	FL (95%)[b]		LC_{50} Expected[c]	Synergism factor[d]	Potency	FL (95%)[b]	
	Slope±SE	Intercept±SE	ng/ml	Lower	Upper	ng/ml			Lower	Upper
Cyt2Ba	0.59±0.13	-3.33±0.79	279.37	190.20	410.38	-	-	1	-	-
Cry10Aa	0.74±0.09	-4.26±0.53	299.62	245.06	366.34	-	-	0.93	0.78	1.12
Cry10Aa+Cyt2Ba[e]	0.45±0.05	-0.64±0.14	4.22	3.25	5.50	144.58	34.26	66.20	58.52	74.61

(a) spore-crystal mixture
(b) FL: Fiducial limits
(c) Expected LC_{50} calculated by the method of Tabashnik (1992)
(d) Synergism factor was defined as the ratio of the expected LC_{50} and the observed LC_{50}
(e) Toxins were present in equal amounts in the experimental inoculum

4. DISCUSSION

The δ-endotoxins that constitute the major parasporal crystal components of Bti strains (Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa) are among the best-studied of the Bt crystal proteins, both in terms of the insecticidal properties of individual proteins and the interactions among them in the digestive tract of susceptible mosquito species. The present study provides evidence that additional proteins, such as Cry10Aa and Cyt2Ba, which are usually present as minor components in Bti strains, are also important toxicity factors that act in a highly synergistic manner when these proteins simultaneously coinfect *A. aegypti* larvae.

When a recombinant Bt strain is constructed for the purpose of producing a particular protein, it can be expected that the expression levels of said protein may be very variable. Initial attempts to express the Cry10Aa protein through the construction of recombinant Bt strains, were unable to produce parasporal crystals (Garduno et al., 1988; Hughes et al., 2005). However, more recently, Hernández-Soto et al (2009) provided evidence of high levels of expression of recombinant Cry10Aa, probably a result of cloning the whole operon including *orf1-orf2*, the inclusion of the cyt1A promoter and the STAB-SD stabilizing sequence in the recipient plasmid, and also the lack of expression competence in the recombinant strain (Hernández-Soto et al., 2009).

The insecticidal activity of Cry10Aa in fourth instar larvae of *A. aegypti* was previously estimated at 2061 ng/ml (Hernández-Soto et al., 2009) which is about 6.87 times upper than the value that we estimated in second instars of the same species. A decrease in the susceptibility of larvae to infection by pathogens with increasing stage is common in insects (Eldefrawi et al., 1964; Stiles and Paschke, 1980), including their susceptibility to Bt toxins (McLaughlin et al., 1984; Otieno-Ayayo et al., 2008; Zehnder and Gelernter, 1989).

Several previous studies have described the larvicidal activity of Cyt2Ba protein in mosquito species belonging to the genera *Culex*, *Aedes* and *Anopheles*, although for a given toxin concentration the observed mortality was lower than that produced by the Cyt1Aa protein, at least for *A. aegypti* larvae (Juárez-Pérez et al., 2002). The estimated value of the LC_{50} value of Cyt2Ba in second instars of *A. aegypti* was approximately 27 times lower than the value estimated by others (Wirth et al., 2001).

These differences may be due, again, to the effect of various factors being the most probable the origin of the population as well as the larval instar used in both laboratories.

The high potential of Bti proteins against mosquito larvae is mainly attributed to the interactions that occur among the component toxins. Cry10Aa and Cyt2Ba, two minor crystal toxins of Bti, contribute to the insecticidal activity of Bti by synergistic interactions (Ben-Dov, 2014). Cry10Aa shows synergistic activity with Cyt1Aa (Hernández-Soto et al., 2009) and Cry4Aa (Delécluse et al., 1988), while Cyt2Ba shows synergistic interaction with Cry4Aa (Manasherob et al., 2006b) and *L. sphaericus* (Wirth et al., 2001) in *A. aegypti*. Although both toxins appear to be weakly expressed in Bti (Ben-Dov, 2014; Guerchicoff et al., 1997; Thorne et al., 1986), when large amounts of these proteins were produced in an acrystalliferous Bt strain, very high levels of toxicity against *A. aegypti* second instar larvae were obtained.

The LC_{50} value obtained for Cry10Aa:Cyt2Ba mixture was about 19 times lower than the value reported for Cry10Aa:Cyt1Aa mixture. This degree of potentiation is one of the highest observed so far for Bti crystal proteins, only comparable to the synergies described for Cyt1A with Cry4Aa and Cry11Aa (Khasdan et al., 2001) or Cry4Ba in mixtures with Cyt2Aa2 from Bt *darmstadiensis* against *A. aegypti* larvae (Promdonkoy et al., 2005) It seems therefore that Cyt2 proteins have a greater involvement in toxin synergy than has been attributed to date.

The toxicity of the Cry10Aa and Cyt2Ba proteins was also tested in their crystallized form and once solubilized, against adults of *Ceratitis capitata*. In our experimental conditions, neither of the two proteins produced mortality either as crystals or following solubilization. Previously, solubilised Cyt1Aa protein from Bti was shown to be toxic to *Ceratitis capitata* larvae (Vidal-Quist et al., 2010). We have not found any activity with the Cyt2Ba protein or the synergistic mixture (Cry10Aa+Cyt2Ba) after basic solubilisation, probably because our assays were performed with adult flies or perhaps because Cyt1Aa and Cyt2Ba have different host ranges.

We conclude that the toxicities of Cry10Aa and Cry2Ba against the mosquito are comparable to the major toxins of Bti and show one of the strongest potentiation effects observed for the Bti crystal

components to date. Further study of the minor crystal components of Bti is likely to provide additional and new opportunities for the development of safe and effective tools for the biological control of mosquitoes of medical importance.

5. REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Becker, M., Ludwig, N., 1993. Investigations on possible resistance in *Aedes Vexans* field populations after a 10-year application of *Bacillus thuringiensis israelensis*. J. Am. Mosq. Control Assoc. 9, 221–224.

Ben-Dov, E., 2014. *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. Toxins (Basel). 6, 1222–1243. https://doi.org/10.3390/toxins6041222

Berry, C., Ben-dov, E., Jones, A.F., Murphy, L., Quail, M. a, Holden, M.T.G., Harris, D., Zaritsky, A., Parkhill, J., 2002. Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 68, 5082–5095. https://doi.org/10.1128/AEM.68.10.5082

Borodovsky, M., McIninch, J., 1993. GENMARK: Parallel gene recognition for both DNA strands. Comput. Chem. 17, 123–133. https://doi.org/10.1016/0097-8485(93)85004-V

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Crickmore, N., Bone, E.J., Williams, J.A., Ellar, D.J., 1995. Contribution of the individual components of the δ-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. FEMS Microbiol. Lett. 131, 249–254. https://doi.org/10.1016/0378-1097(95)00264-6

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Dean, D.H., 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62, 807–13.

Cristian Vidal-Quist, J., Castañera, P., González-Cabrera, J., 2010. Cyt1Aa protein from *Bacillus thuringiensis* (Berliner) serovar *israelensis* is active against the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). Pest Manag. Sci. 66, 949–955. https://doi.org/10.1002/ps.1965

Delécluse, A., Bourgouin, C., Klier, A., Rapoport, G., 1988. Specificity of action on mosquito larvae of *Bacillus thuringiensis israelensis* toxins encoded by two different genes. MGG Mol. Gen. Genet. 214, 42–47. https://doi.org/10.1007/BF00340177

Dominguez-Arrizabalaga, M., Villanueva, M., Fernandez, A.B., Caballero, P., 2019. A strain of *Bacillus thuringiensis* containing a novel *cry7Aa2* gene that is toxic to *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae). Insects 10, 259. https://doi.org/doi:10.3390/insects10090259

Eldefrawi, M.E., Toppozada, A., Mansour, N., Zeid, M., 1964. Toxicological studies on the egyptian cotton leafworm, *Prodenia litura*. I. Susceptibility of different larval instars of Prodenia to insecticides. J. Econ. Entomol. 57, 591–593. https://doi.org/10.1093/jee/57.4.591

Federici, B. a, Park, H., Bideshi, D.K., 2010. Overview of the basic biology of *Bacillus thuringiensis* with emphasis on genetic engineering of bacterial larvicides for mosquito control. Open Toxicol J. 3, 154–171.

Fillinger, U., Lindsay, S.W., 2006. Suppression of exposure to malaria vectors by an order of magnitude using microbial larvicides in rural Kenya. Trop. Med. Int. Heal. 11, 1629–1642. https://doi.org/10.1111/j.1365-3156.2006.01733.x

Garduno, F., Thorne, L., Walfield, A.M., Pollock, T.J., 1988. Structural relatedness between mosquitocidal endotoxins of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 54, 277–279.

Goldberg, L.J., Margalit, J., 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univitattus*, *Aedes aegypti* and *Culex pipiens*. 1, 355–362.

Guerchicoff, A., Ugalde, R.A., Rubinstein, C.P., 1997. Identification and characterization of a previously undescribed cyt gene in *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 63, 2716–2721. https://doi.org/10.1016/j.sse.2014.01.001

Hernández-Soto, A., Del Rincón-Castro, M.C., Espinoza, A.M., Ibarra, J.E., 2009. Parasporal body formation via overexpression of the Cry10Aa toxin of *Bacillus thuringiensis* subsp. *israelensis*, and Cry10Aa-Cyt1Aa synergism. Appl. Environ. Microbiol. 75, 4661–4667. https://doi.org/10.1128/AEM.00409-09

Hughes, P.A., Stevens, M.M., Park, H.W., Federici, B.A., Dennis, E.S., Akhurst, R., 2005. Response of larval *Chironomus tepperi* (Diptera: Chironomidae) to individual *Bacillus thuringiensis* var. *israelensis* toxins and toxin mixtures. J. Invertebr. Pathol. 88, 34–39. https://doi.org/10.1016/j.jip.2004.10.004

Ibarra, J E., Federici, B.A., 1986. Parasporal bodies of *Bacillus thuringiensis* subsp. *morrisoni* (PG-14) and *Bacillus thuringiensis* subsp. *israelensis* are similar in protein composition and toxicity. FEMS Microbiol. Lett. 34, 79–84.

Juárez-Pérez, V., Guerchicoff, A., Rubinstein, C., Delecluse, A., 2002. Characterization of Cyt2Bc toxin from *Bacillus thuringiensis* subsp. *medellin*. Appl. Environ. Microbiol. 68, 1228–1231. https://doi.org/10.1128/AEM.68.3.1228

Khasdan, V., Ben-Dov, E., Manasherob, R., Boussiba, S., Zaritsky, A., 2001. Toxicity and synergism in transgenic *Escherichia coli* expressing four genes from *Bacillus thuringiensis* subsp. *israelensis*. Environ. Microbiol. 3, 798–806. https://doi.org/10.1046/j.1462-2920.2001.00253.x

Krawczyk, P.S., Lipinski, L., Dziembowski, A., 2018. PlasFlow: predicting plasmid sequences in metagenomic data using genome signatures. Nucleic Acids Res. 46, e35. https://doi.org/10.1093/nar/gkx1321

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 225, 680–685.

Lee, J.C., 1995. Electrotransformation of Staphylococci. Electroporation Protoc. Microorg. 47, 209–216. https://doi.org/10.1385/0-89603-310-4:209

Lee, S.G., Eckblad, W., Lee, A.B., 1985. Diversity of protein inclusion bodies and identification of mosquitocidal protein in *Bacillus thuringiensis* subsp. *israelensis*. Biochem. Biophys. Res. Commun. 126, 953–960.

Li, L., Yang, C., Liu, Z., Li, F., Yu, Z., 2000. Screening of acrystalliferous mutants from *Bacillus thuringiensis* and their transformation properties. Wei Sheng Wu Xue Bao 40, 85–90.

Manasherob, R., Itsko, M., Sela-Baranes, N., Ben-Dov, E., Berry, C., Cohen, S., Zaritsky, A., 2006. Cyt1 Ca from *Bacillus thuringiensis* subsp. *israelensis*: Production in *Escherichia coli* and comparison of its biological activities with those of other Cyt-like proteins. Microbiology 152, 2651–2659. https://doi.org/10.1099/mic.0.28981-0

Margalith, Y., and Ben-Dov, E., 2000. Biological control by *Bacillus thuringiensis* subsp. *israelensis*. In Insect Pest Management: Techniques for Environmental Protection. Recheigl, J.E., and Recheigl, N.A.

McLaughlin, R.E., Dulmage, H.T., Alls, R., Couch, T.L., Dame, D.A., Hall, I.M., Rose, R.I., Versoi, P.L., 1984. U.S. Standard bioassay for the potency assessment of *Bacillus thuringiensis* Serotype H-14 against mosquito larvae. Bull. Entomol. Soc. Am. 30, 26–29. https://doi.org/10.1093/besa/30.1.26

Otieno-Ayayo, Z.N., Zaritsky, A., Wirth, M.C., Manasherob, R., Khasdan, V., Cahan, R., Ben-Dov, E., 2008. Variations in the mosquito larvicidal activities of toxins from *Bacillus thuringiensis* ssp. *israelensis*. Environ. Microbiol. 10, 2191–2199. https://doi.org/10.1111/j.1462-2920.2008.01696.x

Park, H.W., Ge, B., Bauer, L.S., Federici, B.A., 1998. Optimization of Cry3A yields in *Bacillus thuringiensis* by use of sporulation-dependent promoters in combination with the STAB-SD mRNA sequence. Appl. Environ. Microbiol. 64, 3932–3938.

Poncet, S., Delecluse, A., Klier, A., Rapoport, G., 1995. Evaluation of synergistic interactions among CryIVA, CryIVB, and CryIVD toxic components of *B. thuringiensis* subsp. *israelensis* crystals. J. Invertebr. Pathol. 66, 131–135.

Promdonkoy, B., Promdonkoy, P., Panyim, S., 2005. Co-expression of *Bacillus thuringiensis* Cry4Ba and Cyt2Aa2 in *Escherichia coli* revealed high synergism against *Aedes aegypti* and *Culex quinquefasciatus* larvae. FEMS Microbiol. Lett. 252, 121–126. https://doi.org/10.1016/j.femsle.2005.08.038

Sambrook, J., Russell, D., 2001. Molecular cloning: a laboratory manual, 3rd ed. ed. Cold Spring Harbor Laboratory, New York.

Stewart, S.D., Adamczyk, J.J., Knighten, K.S., Davis, F.M., 2001. Impact of Bt cottons expressing one or two insecticidal proteins of *Bacillus thuringiensis* Berliner on growth and survival of Noctuid (Lepidoptera) larvae. J. Econ. Entomol. 94, 752–760. https://doi.org/10.1603/0022-0493-94.3.752

Stiles, B., Paschke, J.D., 1980. Midgut pH in different instars of three Aedes mosquito species and the relation between pH and susceptibility of larvae to a nuclear polyhedrosis virus. J. Invertebr. Pathol. 35, 58–64. https://doi.org/10.1016/0022-2011(80)90084-1

Tabashnik, B.E., 1992. Evaluation of synergism among *Bacillus thuringiensis* toxins. Appl. Environ. Microbiol. 58, 3343–3346.

Thorne, L., Garduno, F., Thompson, T., Decker, D., Zounes, M., Wild, M., Waldfield, A., Pollock, T.J., 1986. Structural similarity between the Lepidoptera- and Diptera-specific insecticidal endotoxin genes of *Bacillus thuringiensis* subsp. "*kurstaki*" and "*israelensis*". J. Bacteriol. 166, 801–811. https://doi.org/10.1128/jb.166.3.801-811.1986

Valtierra-de-Luis, Daniel., Villanueva, M., Caballero, Javier., Matas, Isabel., Williams, Trevor., Caballero, Primitivo. 2019. Quantification of dose-mortality responses in adult Diptera: Validation using *Ceratitis capitata* and *Drosophila suzukii* responses to spinosad. Plos One 14(2): e0210545. https://doi.org/10.1371/journal.pone.0210545.

Vasquez, M.I., Violaris, M., Hadjivassilis, A., Wirth, M.C., 2009. Susceptibility of *Culex pipiens* (Diptera: Culicidae) field populations in Cyprus to conventional organic insecticides, *Bacillus thuringiensis* subsp. *israelensis*, and methoprene. J. Med. Entomol. 46, 881–887. https://doi.org/10.1603/033.046.0421

Wirth, M.C., Delecluse, A., Walton, W.E., 2001. Cyt1Ab1 and Cyt2Ba1 from *Bacillus thuringiensis* subsp. *medellin* and *B. thuringiensis* subsp. *israelensis* synergize *Bacillus sphaericus* against *Aedes aegypti* and Resistant *Culex quinquefasciatus* (Diptera: Culicidae). Appl. Environ. Microbiol. 67, 3280–3284. https://doi.org/10.1128/AEM.67.7.3280

Wirth, M.C., Georghiou, G.P., Malik, J.I., Abro, G.H., 2000. Laboratory selection for resistance to *Bacillus sphaericus* in *Culex quinquefasciatus* (Diptera: Culicidae) from California, USA . J. Med. Entomol. 37, 534–540. https://doi.org/10.1603/0022-2585-37.4.534

Wirth, M.C., Park, H.W., Walton, W.E., Federici, B.A., 2005. Cyt1A of *Bacillus thuringiensis* delays evolution of resistance to Cry11A in the mosquito *Culex quinquefasciatus*. Appl. Environ. Microbiol. 71, 185–189. https://doi.org/10.1128/AEM.71.1.185-189.2005

Wu, D., Johnson, J.J., Federici, B.A., 1994. Synergism of mosquitocidal toxicity between CytA and CrylVD proteins using inclusions produced from cloned genes of *Bacillus thuringiensis*. Mol. Microbiol. 13, 965–972. https://doi.org/10.1111/j.1365-2958.1994.tb00488.x

Zapata, N., Budia, F., Viñuela, E., Medina, P., 2006. Insecticidal effects of various concentrations of selected extractions of *Cestrum parqui* on adult and immature *Ceratitis capitata*. J. Econ. Entomol. 99, 359–365.

Zehnder, G.W., Gelernter, W.D., 1989. Activity of the M-ONE Formulation of a new strain of *Bacillus thuringiensis* against the colorado potato beetle (Coleoptera: Chrysomelidae): relationship between susceptibility and insect life stage. J. Econ. Entomol. 82, 756–761. https://doi.org/10.1093/jee/82.3.756

CHAPTER IV

General discussion

Bacterial pesticides, which constitute a series of leading products made from naturally occurring or genetically modified insecticidal bacteria, have attracted increasing attention as a specific means of controlling agricultural, forestry and sanitary pests. Organic insecticides are highly effective but carry a series of side effects, such as harmful effects on wildlife and beneficial non-target insects, contamination of water and food sources, and in the end, the appearance of insecticide-related resistances due to their large-scale use (Carson et al., 2002; Fishel, 2016; Jarman and Ballschmiter, 2012; Tabashnik, 2008; WHO, 1990). In recent years, there has been an increase in alternatives to chemical pesticides, leading to microbial agents, which have proven their efficacy, specificity and safety in use (Chandler et al., 2011).

Bt is the most economical microbial insecticide in terms of production costs. Although a very efficient fermentation process of Bt is required, its production is competitive against synthetic insecticides. Bt has been successfully used for decades to control agricultural pests and human disease vectors like mosquitos, because it is an environmentally friendly and highly specific biopesticide (Bravo et al., 2011; Kamareddine, 2012; Promdonkoy et al., 2005a). The specific toxicity of crystal proteins against target insects is the basis for the use of Bt as a biopesticide in agriculture, forestry and mosquito control since 1961 (Kaur, 2000). The advantages of Bt over synthetic pesticides include lack of polluting residues, high specificity to target insects, safety to non-target organisms such as mammals, birds, amphibians and reptiles, as well as low costs of development and registration (Kaur, 2000).

Bt proteins have been shown to be toxic to a wide variety of insects orders such as Lepidoptera, Coleoptera and Diptera (Salehi Jouzani et al., 2008). Within Bt, there is a large amount of subsp., consisting of different types of strains and serotypes, such as *israelensis*, *jegathesan*, *darmstadiensis*, *kyushensis*, *medellin*, *fukuokaensis*, *higo* etc. whose activity has been mainly referred to dipteran pests.

To evaluate the toxicity of novel insecticidal compounds (strains or proteins) for pest management, simple and repeatable laboratory bioassays methods are required to determine the presence of resistance traits in pest populations (Robertson et al., 2017). The most suitable

bioassay methods usually reflect the mode of action of the toxicant and the biology of the pest. Established assay methods to analyse concentration-mortality responses of pests under controlled laboratory conditions are mainly based on direct topical application, spray droplet delivery systems or contact with residues on treated surfaces (Brodsgaard, 1994; Crowder et al., 1979). Many of the latest generation of insecticidal products that have a selective toxicity spectrum are active by ingestion. Examples include naturally derived products such as spinosad, avermectins or Bt and synthetic compounds such as neonicotinoids and dinamides, among others (Nauen et al., 2012). The accurate quantification of toxicity requires the ability to deliver a known quantity of toxicant onto, or into, the pest. For flies, precise delivery of toxicants by ingestion is more challenging as many feed on liquids that are taken into the crop before being gradually released into the midgut for digestion and assimilation (Stoffolano and Haselton, 2013). Moreover, quantification of the concentration-mortality response requires a method for distinguishing flies that have fed on the toxicant solution from those that have not. This can be achieved by mixing the toxicant with an inert food colored liquid (Baudier et al., 2014). In contrast, dose-mortality studies require quantification of the volume of toxicant solution ingested by experimental flies.

Quantification of ingestion by dipterans is an issue that has received considerable attention, mainly because *Drosophila melanogaster* has become a laboratory model for studies on physiology, nutrition and longevity for which precise estimates of feeding rates are required (Wong et al., 2009). The quantitative methods developed to date include the use of calibrated capillary feeding tubes (the CAFÉ system) (Ja et al., 2007), recording the proboscis extension feeding response (Ja et al., 2007; Mair et al., 2005; Wong et al., 2009), and the use of food in droplets mixed with dye (Edgecomb et al., 1994) or radioactive tracer compounds (Carvalho et al., 2005; Geer et al., 1970) Each of these methods has advantages and drawbacks, but for studies over short periods, the use of radiolabelled tracers in droplets of food has been shown to be highly sensitive, consistent and compatible with natural feeding behaviour in Drosophila (Deshpande et al., 2014). This aspect of feeding on droplets is likely to extent to other dipterans such as tephritids

that feed on droplets of honeydew, fruit juices or bird droppings in the natural environment (Hendrichs and Hendrichs, 1990; Jácome et al., 1999; Manrakhan and Lux, 2006). From a logistical standpoint, once calibrated, the droplet-feeding assay is also quick and easy to set up and the presence of a visible dye in the mixture allows individuals that feed on toxicant droplets to be readily distinguished from those that did not. Quantification of ingestion of a sucrose solution containing ^{32}P-labeled ATP revealed that *C. capitata* males and females ingested an average of 1.74 and 2.19µl of solution respectively, whereas *D. suzukii* consumed 0.69 and 0.98µl for each sex, respectively. This information was used to estimate lethal dose response of both species to the naturally derived insecticide spinosad, from lethal concentration response studies. The 50% lethal dose (LD_{50}) of C. capitata to spionsad was approximately 7-fold more susceptible to spinosad than D. suzukii (based on values of 0.27 and 1.84 ng a.i./mg body weight, respectively).

There are numerous sources of variation in insect bioassay involving ingestion of toxins or pathogens that have to be managed to control overdispersion in the results (Navon and Ascher, 2000; Robertson et al., 2017). In the case of dipterans, these include individual-level effects, such as sporadic or irregular feeding patterns over time, variation in meal size, and body size, developmental and physiological effects. Genetically distinct strains of insects are also expected to feed and respond differently to a given inoculum or toxicant (Kaun et al., 2007; Robertson et al., 1995), particularly if they have a history of exposure to a given compound (Jin et al., 2011). Much of the variation present in insect bioassays can be controlled by standardizing insect rearing conditions (diet, density, temperature, etc.), selecting individuals of the same age and developmental stage, limiting the time available for ingestion of the toxicant to avoid repeated feeding events and using visible dyes to identify individuals that have not engaged in feeding on toxicant droplets. These were precisely the steps that we adopted to minimize variation in our bioassays.

The period during which experimental droplets can be ingested, 20 min in the case of our work, is relevant as excretion begins approximately 40 mins after feeding in Drosophila (Wong et al., 2009), which would have adversely affected the quantification of ingested volumes in the radioactive tracer study. Fortunately, the steps that we took to standardize experimental insect selection and dosing procedures resulted in low levels of variation within assays and among assays involving different batches of insects in the case of *C. capitata*. This type of droplet feeding assay can be readily calibrated to quantify the susceptibility of other species of pestiferous Diptera to insecticidal compounds or pathogens that act by ingestion, such as *Bacillus thuringiensis* or some viruses (Cossentine et al., 2016; Gravot et al., 2000; Habayeb et al., 2009; Prompiboon et al., 2010; Vidal-Quist et al., 2009). The method is simple and reproducible, therefore it could be a very interesting tool when carrying out screening programs for Bt strains with possible insecticidal activity against Diptera.

Due to the importance that Bt-based products have been acquiring to deal with all types of pests replacing chemical pesticides, the major alternative for mosquito and blackfly larval control is based on bacterial toxins produced by Bti. Bti was the first Bt serotype found to be toxic for dipteran species (Goldberg and Margalit, 1977) and products based on that specific bacterium are marketed and are widely used in the US and Europe. Indeed, Bti has been used to control mosquitoes for more than 35 years without any reports of resistance in vector populations (Becker and Ludwig, 1993; Vasquez et al., 2009). The absence of resistance is likely due to the varying modes of action and the synergistic effects of the multiple crystal proteins present in Bti-based products (Crickmore et al., 1995a; Poncet et al., 1995; Wirth et al., 2005). The parasporal crystal of Bti contains large amounts of four toxins: Cry4A, Cry4B, Cry11A and Cyt1A (Ibarra and Federici, 1986). In addition, Cry10Aa and Cyt2Ba have also been described in some Bti strains although these are expressed and accumulated in the crystal in much smaller quantities that the four main components (Garduno et al., 1988; Lee et al., 1985). The high potential of Bti proteins against mosquito larvae is mainly attributed to the interactions that occur among the component

toxins (Otieno-Ayayo et al., 2008). Those involving the Cyt1A protein have attracted particular attention given the capacity of this protein to enhance the insecticidal activity of Cry proteins in strains of Bti (Crickmore et al., 1995b; Hernández-Soto et al., 2009; Wu et al., 1994), and those of Bt strains belonging to other serovars (Wirth et al., 2000).

Cry10Aa and Cyt2Ba, two minor crystal toxins of Bti, contribute to the insecticidal activity of Bti by synergistic interactions (Ben-Dov, 2014). Cry10Aa shows synergistic activity with Cyt1Aa (Hernández-Soto et al., 2009) and Cry4Aa (Delécluse et al., 1988), while Cyt2Ba shows synergistic interaction with Cry4Aa (Manasherob et al., 2006) and *L. sphaericus* (Wirth et al., 2001) in *A. aegypti*. Although both toxins appear to be weakly expressed in Bti (Ben-Dov, 2014; Guerchicoff et al., 1997; Thorne et al., 1986), when large amounts of these proteins were produced in an acrystalliferous Bt strain, very high levels of toxicity against *A. aegypti* second instar larvae were obtained (Chapter III). The LC_{50} value obtained for Cry10Aa:Cyt2Ba mixture was about 19 times lower than the value described for Cry10Aa:Cyt1Aa mixture (Chapter III). This degree of potentiation is one of the highest observed so far for Bti crystal proteins, only comparable to the synergies described for Cyt1A with Cry4Aa and Cry11Aa (Khasdan et al., 2001) or Cry4Ba in mixtures with Cyt2Aa2 from Bt *darmstadiensis* against *A. aegypti* larvae (Promdonkoy et al., 2005b) It seems therefore that Cyt2 proteins have a greater involvement in toxin synergy than has been attributed to date. In conclusion, the toxicities of Cry10Aa and Cry2Ba against the mosquito are comparable to the major toxins of Bti and show one of the strongest potentiation effects observed for the Bti crystal components to date. Further study of the minor crystal components of Bti is likely to provide additional and new opportunities for the development of safe and effective tools for the biological control of mosquitoes of medical importance.

The toxicity of the Cry10Aa and Cyt2Ba proteins was also tested in their crystallized form and once solubilized, against adults of *Ceratitis capitata* (Chapter III). We carried out the experiments following the droplet method set up in Chapter II. In our experimental conditions, neither of the two proteins produced mortality either as crystals or following solubilization. Previously,

solubilised Cyt1Aa protein from Bti was shown to be toxic to *Ceratitis capitata* larvae (Cristian Vidal-Quist et al., 2010). We have not found any activity with the Cyt2Ba protein or the synergistic mixture (Cry10Aa+Cyt2Ba) after basic solubilisation, probably because our assays were performed with adult flies or perhaps because Cyt1Aa and Cyt2Ba have different host ranges.

The use of quantitative laboratory bioassay methods are required to evaluate the toxicity of novel insecticidal compounds for pest control. The technique that we have developed could be readily employed for determination of the resistance status and dose-mortality responses of putative toxic products in many species of pestiferous Diptera. The serotype Bti has been widely used as a microbial larvicide for the control of many species of mosquitoes and blackflies. The mosquitocidal activity of Bti resides in Cry and Cyt δ-endotoxins present in the parasporal crystal of this pathogen. We have shown that the toxicities of Cry10Aa and Cry2Ba against mosquitoes with medical importance are as important as the major toxins of Bti and they show a very high synergistic interaction when *A. aegypti* larvae ingest them in equal proportions.

REFERENCES

Baudier, K.M., Kaschock-Marenda, S.D., Patel, N., Diangelus, K.L., O'Donnell, S., Marenda, D.R., 2014. Erythritol, a non-nutritive sugar alcohol sweetener and the main component of Truvia®, is a palatable ingested insecticide. PLoS One 9, 1–7. https://doi.org/10.1371/journal.pone.0098949

Becker, M., Ludwig, N., 1993. Investigations on possible resistance in *Aedes vexans* field populations after a 10-year application of *Bacillus thuringiensis israelensis*. J. Am. Mosq. Control Assoc. 9, 221–224.

Ben-Dov, E., 2014. *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. Toxins (Basel). 6, 1222–1243. https://doi.org/10.3390/toxins6041222

Bravo, A., Likitvivatanavong, S., Gill, S.S., Soberon, M., 2011.*Bacillus thuringiensis*: A story of a successful bioinsecticide. Insect Biochem. Mol. Biol. 41, 423–431.

Carson, R., Wilson, E.O., Lear, L.J., 2002. Silent Spring. Houghton Mifflin.

Carvalho, G.B., Kapahi, P., Benzer, S., 2005. Compensatory ingestion upon dietary restriction in *Drosophila melanogaster*. Nat. Methods 2, 813–815. https://doi.org/10.1038/nmeth798

Chandler, D., Bailey, A.S., Mark Tatchell, G., Davidson, G., Greaves, J., Grant, W.P., 2011. The development, regulation and use of biopesticides for integrated pest management. Philos. Trans. R. Soc. B Biol. Sci. 366, 1987–1998. https://doi.org/10.1098/rstb.2010.0390

Cossentine, J., Robertson, M., Xu, D., 2016. Biological activity of *Bacillus thuringiensis* in *Drosophila suzukii* (Diptera: Drosophilidae). J. Econ. Entomol. 109, 1071–1078. https://doi.org/10.1093/jee/tow062

Crickmore, N., Bone, E.J., Williams, J.A., Ellar, D.J., 1995. Contribution of the individual components of the δ-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. FEMS Microbiol. Lett. 131, 249–254. https://doi.org/10.1016/0378-1097(95)00264-6

Cristian Vidal-Quist, J., Castañera, P., González-Cabrera, J., 2010. Cyt1Aa protein from *Bacillus thuringiensis* (Berliner) serovar *israelensis* is active against the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). Pest Manag. Sci. 66, 949–955. https://doi.org/10.1002/ps.1965

Crowder, L.A., Tollefson, M.S., Watson, T.F., 1979. Dosage-mortality studies of synthetic pyrethroids and methyl parathion on the tobacco budworm in central Arizona. J Econ Entomol. 72, 1–3.

Delécluse, A., Bourgouin, C., Klier, A., Rapoport, G., 1988. Specificity of action on mosquito larvae of *Bacillus thuringiensis israelensis* toxins encoded by two different genes. MGG Mol. Gen. Genet. 214, 42–47. https://doi.org/10.1007/BF00340177

Deshpande, S.A., Carvalho, G.B., Amador, A., Phillips, A.M., Hoxha, S., Lizotte, K.J., Ja, W.W., 2014. Quantifying Drosophila food intake: comparative analysis of current methodology. Nat. Methods 11, 535–540. https://doi.org/10.1038/nmeth.2899.

Edgecomb, R.S., Harth, C.E., Schneiderman, A.M., 1994. Regulation of feeding behavior in adult *Drosophila melanogaster* varies with feeding regime and nutritional state. J. Exp. Biol. 197, 215–235.

Fishel, F.M., 2016. Pest management and pesticides : A historical early uses of pesticides and other insect-vectored diseases. IFAS extension-UF 2–6.

Garduno, F., Thorne, L., Walfield, A.M., Pollock, T.J., 1988. Structural relatedness between mosquitocidal endotoxins of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 54, 277–279.

Geer, B.W., Olander, R.M., Sharp, P.L., 1970. Quantification of dietary choline utilization in adult *Drosophila melanogaster* by radioisotope methods. J. Insect Physiol. 16, 33–43. https://doi.org/10.1016/0022-1910(70)90110-1

Goldberg, L.J., Margalit, J., 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univitattus*, *Aedes aegypti* and *Culex pipiens*. 1, 355–362.

Gravot, E., Thomas-Orillard, M., Jeune, B., 2000. Virulence variability of the Drosophila C virus and effects of the microparasite on demographic parameters of the host (*Drosophila melanogaster*). J. Invertebr. Pathol. 75, 144–151. https://doi.org/10.1006/jipa.1999.4913

Guerchicoff, A., Ugalde, R.A., Rubinstein, C.P., 1997. Identification and characterization of a previously undescribed

cyt gene in *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 63, 2716–2721. https://doi.org/10.1016/j.sse.2014.01.001

Habayeb, M.S., Cantera, R., Casanova, G., Ekström, J.O., Albright, S., Hultmark, D., 2009. The Drosophila Nora virus is an enteric virus, transmitted via feces. J. Invertebr. Pathol. 101, 29–33. https://doi.org/10.1016/j.jip.2009.02.003

Hendrichs, J., Hendrichs, M.A., 1990. Mediterranean fruit fly (Diptera: Tephritidae) in Nature: location and diel pattern of feeding and other activities on fruiting and nonfruiting hosts and nonhosts. Ann. Entomol. Soc. Am. 83, 632–641. https://doi.org/10.1093/aesa/83.3.632

Hernández-Soto, A., Del Rincón-Castro, M.C., Espinoza, A.M., Ibarra, J.E., 2009. Parasporal body formation via overexpression of the Cry10Aa toxin of *Bacillus thuringiensis* subsp. *israelensis*, and Cry10Aa-Cyt1Aa synergism. Appl. Environ. Microbiol. 75, 4661–4667. https://doi.org/10.1128/AEM.00409-09

Ibarra, J.E., Federici, B.A., 1986. Isolation of a relatively nontoxic 65-kilodalton protein inclusion from the parasporal body of *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. 165, 527–533. https://doi.org/10.1128/jb.165.2.527-533.1986

Ja, W.W., Carvalho, G.B., Mak, E.M., De La Rosa, N.N., Fang, A.Y., Liong, J.C., Brummel, T., Benzer, S., 2007. Prandiology of Drosophila and the CAFE assay. Proc. Natl. Acad. Sci. U. S. A. 104, 8253–8256. https://doi.org/10.1073/pnas.0702726104

Jácome, I., Aluja, M., Liedo, P., 1999. Impact of adult diet on demographic and population parameters of the tropical fruit fly *Anastrepha serpentina* (Diptera: Tephritidae) . Bull. Entomol. Res. 89, 165–175. https://doi.org/10.1017/s0007485399000255

Jarman, W.M., Ballschmiter, K., 2012. From coal to DDT: The history of the development of the pesticide DDT from synthetic dyes till silent Spring. Endeavour 36, 131–142. https://doi.org/10.1016/j.endeavour.2012.10.003

Jin, T., Zeng, L., Lin, Y., Lu, Y., Liang, G., 2011. Insecticide resistance of the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), in mainland China. Pest Manag. Sci. 67, 370–376. https://doi.org/10.1002/ps.2076

Kamareddine, L., 2012. The biological control of the malaria vector. Toxins (Basel). 4, 748–767. https://doi.org/10.3390/toxins4090748

Kaun, K.R., Riedl, C.A.L., Chakaborty-Chatterjee, M., Belay, A.T., Douglas, S.J., Gibbs, A.G., Sokolowski, M.B., 2007. Natural variation in food acquisition mediated via a Drosophila cGMP-dependent protein kinase. J. Exp. Biol. 210, 3547–3558. https://doi.org/10.1242/jeb.006924

Kaur, S., 2000. Molecular approaches towards development of novel *Bacillus thuringiensis* biopesticides. World J. Microbiol. Biotechnol. 16, 781–793. https://doi.org/10.1023/A:1008931207374

Khasdan, V., Ben-Dov, E., Manasherob, R., Boussiba, S., Zaritsky, A., 2001. Toxicity and synergism in transgenic *Escherichia coli* expressing four genes from *Bacillus thuringiensis* subsp. *israelensis*. Environ. Microbiol. 3, 798–806. https://doi.org/10.1046/j.1462-2920.2001.00253.x

Lee, S.G., Eckblad, W., Lee, A.B., 1985. Diversity of protein inclusion bodies and identification of mosquitocidal protein in *Bacillus thuringiensis* subsp. *israelensis*. Biochem. Biophys. Res. Commun. 126, 953–960.

Mair, W., Piper, M.D.W., Partridge, L., 2005. Calories do not explain extension of life span by dietary restriction in *Drosophila*. PLoS Biol. 3, 1305–1311. https://doi.org/10.1371/journal.pbio.0030223

Manasherob, R., Itsko, M., Sela-Baranes, N., Ben-Dov, E., Berry, C., Cohen, S., Zaritsky, A., 2006. Cyt1 Ca from *Bacillus thuringiensis* subsp. *israelensis*: production in *Escherichia coli* and comparison of its biological activities with those of other Cyt-like proteins. Microbiology 152, 2651–2659. https://doi.org/10.1099/mic.0.28981-0

Manrakhan, A., Lux, S.A., 2006. Contribution of natural food sources to reproductive behaviour, fecundity and longevity of *Ceratitis cosyra* , *C. fasciventris* and *C. capitata* (Diptera: Tephritidae) . Bull. Entomol. Res. 96, 259–268. https://doi.org/10.1079/ber2006421

Nauen, R., Elbert, A., McCaffery, A., Slater, R., Sparks, T.C., 2012. IRAC, insecticide resistance and mode of action classification of insecticides. Mod. Crop Prot. Compd. 3, 935–956. https://doi.org/10.1002/9783527619580.ch24

Navon, A., Ascher, K., 2000. Bioassays of entomopathogenic microbes and nematodes. CABI Publishing, Wallingford,

UK.

Otieno-Ayayo, Z.N., Zaritsky, A., Wirth, M.C., Manasherob, R., Khasdan, V., Cahan, R., Ben-Dov, E., 2008. Variations in the mosquito larvicidal activities of toxins from *Bacillus thuringiensis* ssp. *israelensis*. Environ. Microbiol. 10, 2191–2199. https://doi.org/10.1111/j.1462-2920.2008.01696.x

Poncet, S., Delecluse, A., Klier, A., Rapoport, G., 1995. Evaluation of synergistic interactions among CryIVA, CryIVB, and CryIVD toxic components of *B. thuringiensis* subsp. *israelensis* crystals. J. Invertebr. Pathol. 66, 131–135.

Promdonkoy, B., Promdonkoy, P., Panyim, S., 2005. Co-expression of *Bacillus thuringiensis* Cry4Ba and Cyt2Aa2 in *Escherichia coli* revealed high synergism against *Aedes aegypti* and *Culex quinquefasciatus* larvae. FEMS Microbiol. Lett. 252, 121–126. https://doi.org/10.1016/j.femsle.2005.08.038

Prompiboon, P., Lietze, V.U., Denton, J.S.S., Geden, C.J., Steenberg, T., Boucias, D.G., 2010. *Musca domestica* salivary gland hypertrophy virus, a globally distributed insect virus that infects and sterilizes female houseflies. Appl. Environ. Microbiol. 76, 994–998. https://doi.org/10.1128/AEM.02424-09

Robertson, J.L., Jones, M.M., Olguin, E., Alberts, B., 2017. Bioassays with Arthropods, Third Edit. ed. CRC Press.

Robertson, J.L., Preisler, H.K., Ng, S.S., Hickle, L.A., Gelernter, W.D., 1995. Natural variation: a complicating factor in bioassays with chemical and microbial pesticides. J. Econ. Entomol. 88, 1–10. https://doi.org/10.1093/jee/88.1.1

Salehi Jouzani, G., Pourjan Abad, A., Seifinejad, A., Marzban, R., Kariman, K., Maleki, B., 2008. Distribution and diversity of Dipteran-specific cry and cyt genes in native *Bacillus thuringiensis* strains obtained from different ecosystems of Iran. J. Ind. Microbiol. Biotechnol. 35, 83–94. https://doi.org/10.1007/s10295-007-0269-6

Stoffolano, J.G., Haselton, A.T., 2013. The adult dipteran crop: a unique and overlooked organ. Annu. Rev. Entomol. 58, 205–225. doi:10.1146/annurev-ento-120811-153653

Tabashnik, B.E., 2008. Delaying insect resistance to transgenic crops. Proc. Natl. Acad. Sci. U. S. A. 105, 19029–19030. https://doi.org/10.1073/pnas.0810763106

Thorne, L., Garduno, F., Thompson, T., Decker, D., Zounes, M., Wild, M., Waldfield, A., Pollock, T.J., 1986. Structural similarity between the Lepidoptera- and Diptera-specific insecticidal endotoxin genes of *Bacillus thuringiensis* subsp. "*kurstaki*" and "*israelensis*." J. Bacteriol. 166, 801–811. https://doi.org/10.1128/jb.166.3.801-811.1986

Vasquez, M.I., Violaris, M., Hadjivassilis, A., Wirth, M.C., 2009. Susceptibility of *Culex pipiens* (Diptera: Culicidae) field populations in Cyprus to conventional organic insecticides, *Bacillus thuringiensis* subsp. *israelensis*, and methoprene. J. Med. Entomol. 46, 881–887. https://doi.org/10.1603/033.046.0421

Vidal-Quist, J.C., Castañera, P., González-Cabrera, J., 2009. Diversity of *Bacillus thuringiensis* strains isolated from citrus orchards in Spain and evaluation of their insecticidal activity against *Ceratitis capitata*. J. Microbiol. Biotechnol. 19, 749–759. https://doi.org/10.4014/jmb.0810.595

WHO, 1990. Public health impact of pesticides used in agriculture. Trans. R. Soc. Trop. Med. Hyg.

Wirth, M.C., Delecluse, A., Walton, W.E., 2001. Cyt1Ab1 and Cyt2Ba1 from *Bacillus thuringiensis* subsp. *medellin* and *B. thuringiensis* subsp. *israelensis* Synergize *Bacillus sphaericus* against *Aedes aegypti* and Resistant *Culex quinquefasciatus* (Diptera: Culicidae). Appl. Environ. Microbiol. 67, 3280–3284. https://doi.org/10.1128/AEM.67.7.3280

Wirth, M.C., Georghiou, G.P., Malik, J.I., Abro, G.H., 2000. Laboratory Selection for Resistance to *Bacillus sphaericus* in *Culex quinquefasciatus* (Diptera: Culicidae) from California, USA . J. Med. Entomol. 37, 534–540. https://doi.org/10.1603/0022-2585-37.4.534

Wirth, M.C., Park, H.W., Walton, W.E., Federici, B.A., 2005. Cyt1A of *Bacillus thuringiensis* delays evolution of resistance to Cry11A in the mosquito *Culex quinquefasciatus*. Appl. Environ. Microbiol. 71, 185–189. https://doi.org/10.1128/AEM.71.1.185-189.2005

Wong, R., Piper, M.D.W., Wertheim, B., Partridge, L., 2009. Quantification of food intake in Drosophila. PLoS One 4. https://doi.org/10.1371/journal.pone.0006063

Wu, D., Johnson, J.J., Federici, B.A., 1994. Synergism of mosquitocidal toxicity between CytA and CrylVD proteins using inclusions produced from cloned genes of *Bacillus thuringiensis*. Mol. Microbiol. 13, 965–972. https://doi.org/10.1111/j.1365-2958.1994.tb00488.x

CONCLUSIONS

CONCLUSIONES

1. El método de bioensayo de la gota (*droplet feeding method*), desarrollado por Hughes y Wood en 1981 para larvas de lepidópteros, ha sido adaptado para adultos de dos especies de moscas de la fruta de gran relevancia económica: *Ceratitis capitata* y *Drosophila suzukii*. Para validar dicha adaptación se ha utilizado con éxito como materia activa el plaguicida de origen natural spinosad.
2. Este método de bioensayo es más preciso y reproducible que otros empleados hasta la fecha, ya que permite seleccionar sólo aquellos individuos que han ingerido una dosis de compuesto tóxico adecuada en un intervalo de tiempo inferior a 20 minutos. La selección de los individuos se puede hacer a simple vista gracias a la adición de un colorante a la solución de tratamiento.
3. Se ha determinado experimentalmente que los machos y hembras de *C. capitata* ingieren un volumen medio de 1.74 y 2.19 µL, respectivamente, mientras que los machos y hembras de *D. suzukii* ingieren 0.69 y 0.98 µL, respectivamente.
4. La aplicación del *droplet feeding method* ha permitido determinar que los machos (LC_{50} = 0.840 ug/ml) y las hembras (LC_{50} = 0.687 ug/ml) de *C. capitata* son igualmente susceptibles al plaguicida de origen natural spinosad.
5. El *droplet feeding method* permite estimar el valor de la LD_{50} cuando se conoce la cantidad de ingrediente activo ingerido por el insecto. El valor de la LD_{50} de spinosad como materia activa para *C. capitata* (LD_{50} = 1.423 ng/insecto) es aproximadamente la mitad que para *D. suzukii* (LD_{50} = 2.927 ng/insecto). Sin embargo, cuando el valor de la LD_{50} se calcula teniendo en cuenta el peso corporal del insecto, *C. capitata* es aproximadamente 7 veces más susceptible a spinosad que *D. suzukii*.
6. Se ha demostrado la elevada potencia insecticida de las proteínas Cry10Aa (LC_{50} = 299.62 ng/ml) y Cyt2Ba (LC_{50} = 279.37 ng/ml) contra larvas L_2 de *Aedes aegypti*, pese a tener una representación minoritaria en la composición del cristal en algunas cepas de *B. thuringiensis* ser. *israelensis*.
7. Se ha observado que cuando las proteínas Cry10Aa y Cyt2Ba se suministran a las larvas de *A. aegypti*, de manera conjunta y en la misma proporción relativa (1:1), se produce un importante efecto sinérgico, ya que el valor de la LC_{50} observado (4.22 ng/ml) fue mucho menor que el valor

esperado (144,58 ng/ml) en caso de asumir un efecto aditivo. Este efecto sinérgico es uno de los más potentes de los descritos para larvas de mosquito hasta el momento.

8. Las proteínas Cry10Aa y Cyt2Ba no tienen actividad tóxica contra los adultos de *C. capitata* tanto si son ingeridas de forma individual como si son ingeridas conjuntamente.

CONCLUSIONS

1. The droplet feeding method developed by Hughes and Wood in 1981 for lepidopteran larvae has been adapted for adults of two species of fruit flies of great economic relevance: *Ceratitis capitata* and *Drosophila suzukii*. To validate this adaptation, the naturally-derived insecticide spinosad has been successfully used as active ingredient.
2. This is a more accurate and reproducible bioassay method compared to others employed until date, since it allows to select those individuals which have ingested the appropriate dose of toxic compound in a 20 minutes interval of time. The selection of individuals can be performed due to the addition of a dye to the treatment solution.
3. It has been experimentally determined that males and females of *C. capitata* ingest an average volume of 1.74 and 2.19 µL, respectively, while males and females of *D. suzukii* ingest 0.69 and 0.98 µL, respectively.
4. The application of the droplet feeding method has allowed us to determine that males (LC_{50} = 0.840 ug/ml) and females (LC_{50} = 0.687 ug/ml) of *C. capitata* are equally susceptible to the naturally-derived insecticide spinosad.
5. The droplet feeding method allows to estimate the value of LD_{50} when the amount of active ingredient ingested by the insect is known. The LD_{50} value of spinosad as the active ingredient for *C. capitata* (LD_{50} = 1.423 ng/insect) is approximately half that for *D. suzukii* (LD_{50} = 2.927 ng/insect). However, when the LD_{50} value is calculated taking into account insect's body weight, *C. capitata* is approximately 7 times more susceptible to spinosad than *D. suzukii*.
6. It has been demonstrated the high insecticidal potency of the Cry10Aa (LC_{50} = 299.62 ng/ml) and Cyt2Ba (LC_{50} = 279.37 ng/ml) proteins against *Aedes aegypti* second instars, although their minor representation in the parasporal crystal of some of *B. thuringiensis* ser *israelensis* strains.
7. It has been observed that when the Cry10Aa and Cyt2Ba proteins are supplied to the *A. aegypti* larvae, together and in the same relative proportion (1:1), an important synergistic effect occurs, since the value of the LC_{50} observed (4.22 ng/ml) was much lower than the expected value (144,58 ng/ml) if it assumed an additive effect. This synergistic effect is one of the most potent described for mosquito larvae, so far.

8. The Cry10Aa and Cyt2Ba proteins have no toxic activity against *C. capitata* adults whether they are ingested individually or together.

LIST OF PUBLICATIONS

LIST OF PUBLICATIONS

Daniel Valtierra de Luis, Maite Villanueva, Colin Berry, Primitivo Caballero*. 2019. *Bacillus thuringiensis* insecticidal proteins against Diptera. Toxins, to be submitted

Daniel Valtierra-de-Luis, Maite Villanueva, Javier Caballero, Isabel M. Matas, Trevor Williams*, Primitivo Caballero. 2019. Quantification of dose-mortality responses in adult Diptera: validation using *Ceratitis capitata* and *Drosophila suzukii* responses to spinosad. Plos One, Published

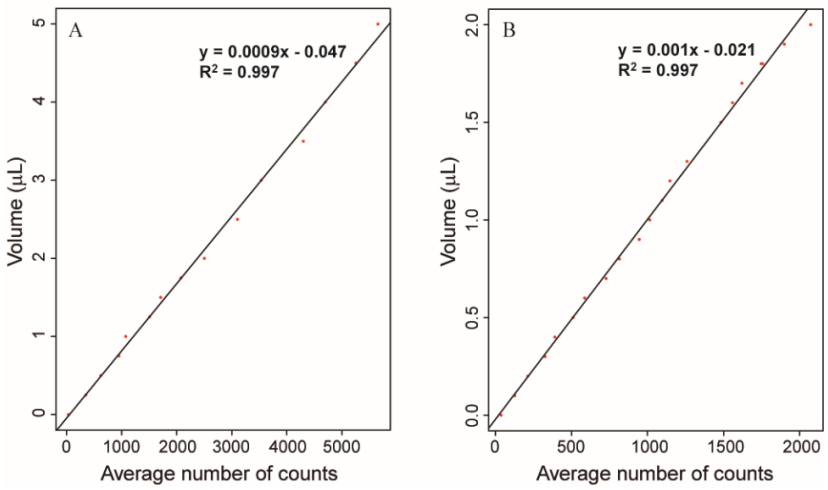
Valtierra-de-Luis, Daniel; Villanueva, Maite; Trevor Williams; and Caballero, Primitivo*. 2019. Synergistic interaction of the δ-endotoxins Cry10Aa and Cyt2Ba from *Bacillus thuringiensis* ser. *israelensis* in *Aedes aegypti* larvae. Pest Management Science, to be submitted

*Corresponding author

SUPPORTING INFORMATION

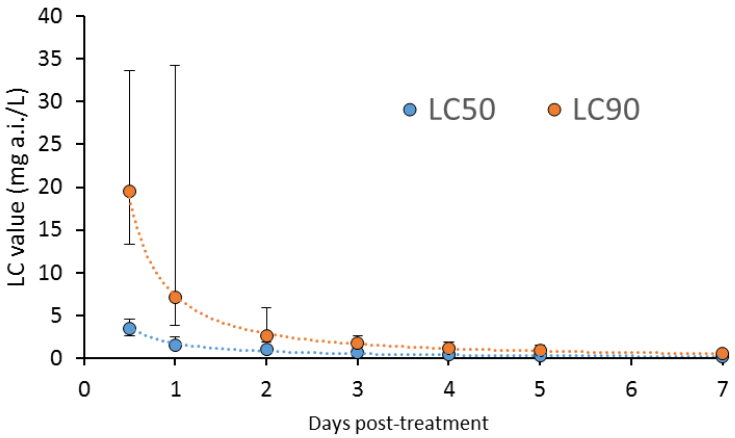
SUPPORTING INFORMATION

S1 Fig 1. Calibration curve for quantification of ingested volume in (A) *Ceratitis capitata* and (B) *Drosophila suzukii*. Adult flies ingested experimental droplets containing 32P -labelled adenosine triphosphate (3000Ci/mmol, PerkinElmer), in mixtures with fluorella blue, hydrolyzed protein and sucrose (see Methods section of text). Red dots indicate the average of five 1-min counts in a scintillation counter plotted against a range of dilutions of ATP-γ-^{32}P. The resulting correlation lines and equations are shown.



S1 Fig 2. Relationship between LC50 (blue points) and LC90 (orange points) values in mg active ingredient (a.i.)/liter at different intervals (0.5 - 7 days) following ingestion of spinosad by

***Ceratitis capitata* adults, as reported by Adan et al. (1995).** Vertical bars are asymetrical and indicate 95% confidence intervals of estimated value.



Source: Adán, A., Estal, P.D., Budia, F., González, M., Viñuela, E., 1996. Laboratory evaluation of the novel naturally derived compound spinosad against *Ceratitis capitata*. Pesticide Science 48, 261-268.